The Influence of Mutualisms Below Ground on Multitrophic Interactions Above Ground

by

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Dedication

To the wild places that sustain and inspire me

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Abstract

Multitrophic species interactions are shaped by a combination of top-down and bottom-up forces. Mutualisms, by altering partner phenotype, may directly and indirectly alter the strength of these forces. However, the ecological consequences of mutualisms on multitrophic interactions, and the mechanisms by which this may occur, are just beginning to be understood. In this dissertation, I combine a series of manipulative experiments to assess the effects of ubiquitous mutualists of plants belowground, arbuscular mycorrhizal fungi (AMF), on multitrophic interactions. First, in Chapter II, I investigated the effects of the availability of AMF on the induction of milkweed (*Asclepias*) defenses by herbivores above and belowground, and how herbivore damage influenced AMF colonization of roots. I found that the relative induction or suppression of foliar cardenolides (chemical defenses) and leaf toughness by herbivores was altered by the level of AMF inoculum available to plants, but AMF did not influence herbivore-induction of root cardenolides. Furthermore, I showed that herbivore feeding altered levels of AMF colonization substantially, completing a feedback loop between above and belowground organisms. Next, in Chapter III, I evaluated how AMF-mediated changes in plant traits affect toxin sequestration and performance of oleander aphids (*Aphis nerii*) and monarch caterpillars (*Danaus plexippus*). Following AMF-mediated increases in cardenolide concentrations, herbivores sequestered higher concentrations of cardenolides from plants inoculated with AMF across all milkweed species; greater sequestration may help to protect herbivores from natural enemies. In addition, aphid per capita growth rates and individual masses varied with AMF availability, consistently among milkweed species. Aphid performance was greatest on plants under high AMF availability, least on plants under medium AMF availability, and intermediate on plants without AMF. In contrast, caterpillar survival varied strongly with AMF availability in a species-specific manner, highlighting the importance of herbivore identity in their responses to AMF. In Chapter IV, I examined how AMF influence constitutive and aphid-induced volatile organic compound (VOC) emissions in two milkweed species, *A. curassavica* and *A. incarnata*. I

found that AMF had species-specific effects on VOC emissions; AMF increased total VOC emissions, green leafy volatiles, and methyl salicylate in *A. curassavica* but decreased these compounds in *A. incarnata*. In contrast, AMF suppressed emissions of individual terpenes that are also suppressed by aphid feeding in both plant species. As these compounds are important to herbivore and natural enemy attraction, these findings suggest that AMF may alter herbivore-natural enemy interactions in the field. In Chapter V, I examined how AMF affect multitrophic interactions in the field. I found that AMF increased the probability of aphid colonization consistently among plant species but altered aphid abundances differentially among plant species. Following AMF-mediated increases in aphid colonization and abundance, total predator abundances were greatest on plants under high AMF availability, consistently among plant species. However, effects of AMF on individual predators varied; colonization by spiders varied with AMF availability differentially among plant species, irrespective of aphid density. In contrast, aphid midge fly oviposition and predation of aphids varied strongly with aphid density and the amount of AMF available to plants. Most notably, the per capita mortality rate imposed by midge flies on aphids varied with AMF availability. Taken together, my research shows that AMF affect strongly both top-down (via toxin sequestration and natural enemy attraction) and bottom-up (via plant defense and nutrition) forces, indicating that AMF may have pervasive effects on multitrophic interactions.

Chapter I
Introduction

1.1 Mutualism in a multitrophic context

Multitrophic species interactions are governed by a combination of top-down forces, such as predators and parasites, and bottom-up forces, such as resource availability (Hunter and Price 1992, Schmitz et al. 2000). Mutualisms, by altering partner phenotype, may directly or indirectly alter the strength of these forces. Mutualisms have strong, but context-dependent, effects on partner phenotypes (Holland et al. 2002, Klironomos 2003, Schulz and Boyle 2005, Anacker et al. 2014). In addition, the effects of mutualisms on partner phenotypes and performance are inherently nonlinear due to the costs that mutualisms exact for the benefits that they provide (Bronstein 2001, Holland et al. 2002, Vannette and Hunter 2011). Therefore, mutualisms may have strong, but complex, effects on multitrophic interactions. Although mutualisms are ubiquitous, the role of mutualisms in shaping multitrophic interactions remains relatively understudied (Holland et al. 2002).

Microbial mutualisms can influence partner phenotype profoundly, with consequences for multitrophic interactions. For example, microbial symbioses with both plants and animals improve nutrient acquisition (Dillon and Dillon 2004, Smith and Read 2008, Tremaroli and Bäckhed 2012) and defense against pathogens and predators (Pozo and Azcón-Aguilar 2007, Kamada et al. 2013, Lewandowski et al. 2013). Furthermore, changes in phenotype in response to symbioses cascade up trophic levels. For instance, grasses infected with foliar endophytic fungi have altered foliar chemistry (Clay 1988), which influences herbivore performance (Gange et al. 2012) and overall arthropod community structure (Omacini et al. 2001). Importantly, because of variation in costs and benefits, "mutualisms" are not always beneficial for both organisms involved, and can range from parasitic to mutualistic depending on environmental conditions and the particular species involved (Johnson et al. 1997, Stadler and Dixon 1998,

Bronstein 2001, Denison and Kiers 2004, Müller and Krauss 2005, Saikkonen et al. 2010, Hoeksema et al. 2010, Johnson and Graham 2013, Afkhami et al. 2014).

In terrestrial ecosystems, both top-down and bottom-up forces travel with ease across the traditional soil "boundary", with plants connecting the interactions that occur between above and belowground organisms (van der Putten et al. 2001, van Dam and Heil 2011, Hunter 2016). As a result, mutualists belowground impact the performance of aboveground insect herbivores from the bottom-up (Erb et al. 2008, Koricheva et al. 2009, Pineda et al. 2010, Rasmann et al. 2017), and the resistance of herbivores to their natural enemies from the top-down (Gange et al. 2003, Rasmann et al. 2017, Tao et al. 2017). Although ubiquitous, the mechanisms and ecological consequences of such interactions are just beginning to be understood (Erb et al. 2009, Papadopoulou and van Dam 2017, De Deyn 2017).

1.2 Arbuscular mycorrhizal fungi

In my dissertation, I evaluate how the ubiquitous mutualists of plants belowground, arbuscular mycorrhizal fungi (AMF), influence multitrophic interactions. AMF make up the monophyletic fungal phylum Glomeromycota (Schüßler et al. 2001) and associate with over eighty percent of plant species globally, including bryophytes, pteridophytes, gymnosperms, and angiosperms (Wang and Qiu 2006, Smith and Read 2008, Soudzilovskaia et al. 2015). AMF colonize the roots of plants and provide plants with nutrients, including phosphorous, nitrogen, zinc, copper and micronutrients, in exchange for plant sugars (Smith and Read 2008). In establishing and maintaining the symbiosis, AMF also interact with plant defensive signaling pathways, including the jasmonic acid and salicylic acid pathways (Jung et al. 2012, Cameron et al. 2013, Gutjahr 2014, Bucher et al. 2014). As a result, AMF alter plant nutritive quality and a diversity of plant primary and secondary metabolites (Bennett et al. 2009, Vannette et al. 2013, Roger et al. 2013, Schweiger et al. 2014, Schweiger and Müller 2015, Hill et al. 2018). In addition, AMF prime plants to defend against attack, leading to greater and more rapid expression of defense genes after damage (Jung et al. 2012, Song et al. 2013, He et al. 2017). The association with AMF is often mutualistic for plants, as AMF frequently stimulate plant growth and mitigate abiotic and pathogen stress (Smith and Read 2008). However, like all mutualisms, the effects of AMF on plant growth and defense range from beneficial to

detrimental, depending on the environment (e.g. Hoeksema et al. 2010), plant and AMF identity (e.g. Klironomos 2003, Tao et al. 2016), and the density of AMF inoculum available to the plant (Garrido et al. 2010, Vannette and Hunter 2011, 2013).

AMF may alter multitrophic interactions from the bottom-up by altering plant quality for herbivores (Hartley and Gange 2009, Koricheva et al. 2009). The response of insect herbivores to AMF colonization of their host plants varies widely, from positive to neutral or negative (Koricheva et al. 2009). Much of this variation is explained by the degree of specialization and feeding mode of the herbivore (Hartley and Gange 2009, Koricheva et al. 2009). For instance, both generalist and specialist phloem-feeding insects, such as aphids, generally benefit from AMF colonization of their host plants. Specialist chewing herbivores, such as caterpillars, also often benefit, but generalist chewing herbivores are negatively affected by AMF colonization of their host plants (Hartley and Gange 2009, Koricheva et al. 2009). Phloem-feeding insects may avoid AMF-mediated increases in plant defenses because phloem lacks or contains far lower concentrations of plant secondary metabolites than do leaves (Züst and Agrawal 2016a). In addition, phloem-feeding insects may benefit from AMF-mediated increases in the size of plant vascular bundles (Krishna et al. 1981, Simon et al. 2017). Generalist chewers may be more susceptible to AMF-mediated increases in plant defenses (Schoonhoven et al. 2005), while specialist chewers may benefit from increased nutritive quality of host plants colonized by AMF (Koricheva et al. 2009). However, even within these trends, there is large variation in herbivore responses to AMF, and we lack an understanding of the specific, AMF-mediated changes in plant phenotype that are driving this variation.

In addition to being shaped by host plant quality, herbivore populations are also affected by their natural enemies (Turchin et al. 2003). AMF may indirectly alter the strength of top-down forces on herbivore populations by altering the resistance of herbivores to their natural enemies. Many specialist herbivores are able to resist their natural enemies by sequestering secondary metabolites from their host plants, making themselves toxic and deterrant to their natural enemies (Nishida 2002, Opitz and Müller 2009, Ode 2013, Erb and Robert 2016, Petschenka and Agrawal 2016). The concentration and composition of secondary metabolites that herbivores sequester are tied closely with host plant secondary chemical profiles (Malcolm

1990, 1994, Agrawal et al. 2015, Petschenka and Agrawal 2015). By altering plant chemical defenses, AMF may mediate toxin sequestration by herbivores, thereby influencing herbivore resistance to their natural enemies.

Furthermore, AMF may mediate the strength of top-down forces on herbivores by altering the attraction of their natural enemies to plants. Plants communicate with and respond to other members of their communities via volatile organic compounds (VOCs) (Kessler and Baldwin 2001, Dicke and Baldwin 2010, Hare 2011, Karban et al. 2014, Rowen and Kaplan 2016, Turlings and Erb 2018). Using VOCs, plants can cue natural enemies to their herbivore prey on plants, reducing damage by herbivores (Kessler and Baldwin 2001, Turlings and Erb 2018). However, herbivores also utilize volatile cues to identify appropriate hosts (Bruce et al. 2005, Bruce and Pickett 2011). AMF, by altering plant nutrient uptake and interacting with plant defensive signaling pathways, can alter the emissions of constitutive and herbivore-induced volatile organic compounds (Rapparini et al. 2008, Fontana et al. 2009, Leitner et al. 2010, Asensio et al. 2012, Schausberger et al. 2012, Babikova et al. 2014b, 2014a, Shrivastava et al. 2015), influencing the attraction of herbivores and their natural enemies (Guerrieri et al. 2004, Schausberger et al. 2012, Babikova et al. 2014b, 2014a). However, most studies to date have been limited to crop plant species, although AMF may alter volatile emissions in wild plant species (but see Fontana et al. 2009).

1.3 Study system - *Asclepias*

In my dissertation, I investigate the influence of AMF on aboveground multitrophic interactions, and evaluate mechanisms by which this may occur. To do so, I use a series of greenhouse and field experiments with milkweed (*Asclepias*) species. Milkweeds provide an ideal system in which to address these questions because milkweeds produce a suite of well-characterized resistance traits in leaves and roots that affect milkweed herbivores substantially (Agrawal 2004, Rasmann et al. 2009a, Rasmann and Agrawal 2011) and vary with AMF colonization (Vannette and Hunter 2011, Vannette et al. 2013, Tao et al. 2016a). In their foliar and root tissues, milkweeds produce cardenolides, bitter tasting steroids that disrupt the functioning of sodium-potassium channels in animal cells (Agrawal et al. 2012). Depending on herbivore identity, herbivore feeding can increase or decrease cardenolide concentrations in

leaves (Zehnder and Hunter 2007, de Roode et al. 2011, Agrawal et al. 2014) and in roots (Rasmann et al. 2009a, 2011, Erwin et al. 2014). In response to leaf damage, milkweeds exude latex, a sticky isoprene polymer that gums up the mouths of chewing herbivores (Zalucki et al. 2001, Agrawal and Konno 2009). Milkweed species also vary in leaf toughness (Agrawal and Fishbein 2006). In addition, milkweed species vary in their sesquiterpene emissions, which correlate with top-down pressure by predators on herbivores in the field (Mooney et al. 2010). Furthermore, common milkweed plants (*A. syriaca*) emit herbivore-induced plant volatiles (HIPVs) that attract natural enemies in response to caterpillar damage, indicating that milkweed species have effective indirect defenses (Wason and Hunter 2014). Therefore, AMF effects on milkweed volatile emissions may result in differences in herbivore and natural enemy attraction in the field.

To assess the effects of AMF on herbivore performance and herbivore-predator interactions, I used two specialist herbivores of milkweed that vary in their feeding mode: oleander aphids (*Aphis nerii*; phloem-feeding) and monarch caterpillars (*Danaus plexippus*; leaf-chewing). Both oleander aphids and monarch caterpillars can tolerate cardenolides, but exhibit reduced performance on host plants with high concentrations of cardenolides (Zalucki et al. 2001a, Agrawal 2004, 2005, Rasmann et al. 2009, de Roode et al. 2011, Colvin et al. 2013, Tao et al. 2016b, Birnbaum et al. 2017). Furthermore, oleander aphids and monarch caterpillars sequester cardenolides (Rothschild et al. 1970, Malcolm and Brower 1989, Malcolm 1990, Züst and Agrawal 2016b), providing some defense against aphid predators (Pasteels 1978, Malcolm 1989, 1992, Pappas et al. 2007, Mooney et al. 2008) and monarch predators and parasites (Brower et al. 1968, Brower and Moffitt 1974, Sternberg et al. 2012). Cardenolide sequestration by both oleander aphids and monarch caterpillars is closely correlated with their host plant cardenolides (Malcolm 1990, 1994, Agrawal et al. 2015, Petschenka and Agrawal 2015). Thus, AMF-mediated changes in plant cardenolide expression may influence aphid and caterpillar sequestration.

To evaluate how AMF affect interactions among plants, herbivores, and their natural enemies, we used a commercial mix of AMF (Mycorrhizal Applications, Grants Pass, OR, USA) that was advertised to contain equal proportions of four common AMF species including

Rhizophagus intraradices, *Funneliformis mosseae*, *Glomus aggregatum*, and *Claroideoglomus etunicatum*. Milkweeds grow in habitats that host a diversity of AMF taxa (Öpik et al. 2006), and can form associations with these cosmopolitan AMF species in natural and experimental populations (Landis et al. 2004, Vannette and Hunter 2011, Vannette et al. 2013, Tao et al. 2015, 2016a). However, as with most systems, the frequency of these relationships in natural populations is not known. It would have been ideal to use specific AMF strains isolated from the roots of all the milkweed species that I included in my experiments, and to investigate multitrophic interactions in the same habitats from which the AMF were isolated. However, because the milkweed species that I used do not all co-occur in the same communities, I would not have been able to separate the confounded effects of milkweed species and AMF strain on multitrophic interactions. Therefore, I chose to use the common commercial inoculum with all milkweed species.

We manipulated the amount of live AMF inoculum available to plants to generate different levels of root colonization. Importantly, the amount of AMF available to plants may affect the ratio of phosphorous benefit to carbon cost for plants (Vannette and Hunter 2011), leading to the expectation of a nonlinear responses in plant phenotype to AMF availability (Vannette and Hunter 2011, 2013). Furthermore, plant regulation of AMF colonization of roots via phytohormones (Staehelin et al. 2011, Gutjahr 2014, Bucher et al. 2014, and references therein) may also affect plant phenotype and responses to herbivores, potentially affecting multitrophic interactions. Although the availability of AMF inoculum varies from landscape (Lekberg and Koide 2005, Soudzilovskaia et al. 2015) to centimeter scales (Wolfe et al. 2007), few studies have considered how the availability of AMF in soils may affect multitrophic interactions (Tao et al. 2015)

1.3 Summary of dissertation chapters

My dissertation is divided into four chapters in which I investigate the effects of AMF on multitrophic interactions, and the mechanisms by which they occur. In Chapter II, I examine how AMF influence the induction of plant defenses by herbivores in both above and belowground plant tissues, and how damage by herbivores feeds back to affect AMF colonization of plant roots. In Chapter III, I evaluate how AMF-mediated changes in plant defenses and nutritive

quality influence toxin sequestration and performance of two specialist herbivores. In Chapter IV, I assess how AMF affect milkweed emissions of constitutive and herbivore-induced volatile organic compounds (VOCs). Lastly, in Chapter V, I examine the ecological relevance of AMF-mediated changes in plant phenotype by exploring the influence of AMF availability on interactions among plants, herbivores, and their predators in the field.

Chapter II: Arbuscular mycorrhizal fungi mediate herbivore-induction of plant defenses differently above and belowground

Plants are exposed to herbivores and symbionts above and belowground. Herbivores aboveground alter plant defenses in both leaves and roots, affecting plant-herbivore interactions above and belowground (Erb et al. 2008, Johnson et al. 2012). Root symbionts, such as arbuscular mycorrhizal fungi (AMF), also influence the defenses of leaves and roots (Vannette et al. 2013, Hill et al. 2018), and alter plant responses to herbivory (Bennett et al. 2009, Kempel et al. 2010, Barber 2013, Babikova et al. 2014, Wang et al. 2015, Minton et al. 2016, He et al. 2017). However, we lack an understanding of how AMF mediate plant responses to herbivores simultaneously in above and belowground plant tissues, despite the prevalence of such interactions. Therefore, in this chapter I evaluate how the availability of AMF influences herbivore-induction of defenses both above and belowground, and how herbivores affect AMF colonization of roots.

In a full factorial experiment, we subjected plants of four milkweed species (*Asclepias incarnata, A. curassavica, A. latifolia*, and *A. syriaca*) under three levels of AMF inoculum availability to damage by aphids (*Aphis nerii*), caterpillars (*Danaus plexippus*), or no herbivores. We then measured foliar and root cardenolides, leaf toughness, latex exudation, foliar carbon, nitrogen, and phosphorous concentrations, plant biomass, and levels of AMF colonization of roots. We predicted that caterpillars would increase plant defenses to varying extents among plant species, whereas aphids would suppress plant defenses. We expected AMF to enhance plant responses to caterpillars, but attenuate plant responses to aphids. Furthermore, we expected the strength of these effects to vary with AMF availability. In contrast, we had no specific predictions for effects of AMF on the induction of root defenses by aboveground herbivores because responses of root traits to shoot herbivory are highly variable among plant and herbivore

species (Erb et al. 2008). Lastly, we predicted that aphid feeding would decrease mycorrhizal colonization, but that short-term caterpillar feeding would increase mycorrhizal colonization. However, because the outcomes of many AMF-plant associations are specific to the AMF and plant species (e.g. Grman 2012, Barber et al. 2013, Anacker et al. 2014, Tao et al. 2016), we expected the strength of these effects to vary among plant species.

Chapter III. Mycorrhizae alter toxin sequestration and performance of two specialist herbivores

As I demonstrate in Chapter II, belowground symbionts of plants, such as arbuscular mycorrhizal fungi (AMF), alter plant phenotype substantially. These changes in plant phenotype may influence herbivore populations from the bottom-up, by influencing herbivore growth and fecundity (Hartley and Gange 2009, Koricheva et al. 2009). In addition, AMF-mediated changes in plant phenotype may affect the top-down forces acting on herbivores by altering toxin sequestration by herbivores; toxin sequestration is tied closely with host plant secondary chemical profiles (Malcolm 1990; Malcolm 1994; Agrawal et al., 2015; Petschenka and Agrawal, 2015). Therefore, in this chapter, I evaluate how AMF influence toxin sequestration and performance of two specialist herbivores feeding upon four milkweed species (*Asclepias incarnata, A. curassavica, A. latifolia,* and *A. syriaca*) by altering plant phenotype.

We raised aphids (*Aphis nerii*) and caterpillars (*Danaus plexippus*) on plants for six days in a fully factorial manipulation of milkweed species and level of AMF availability (zero, medium, and high). We then assessed the performance of aphids and caterpillars, and their levels of cardenolide sequestration. Concurrently, we measured the defensive and nutritive traits of control plants. We expected herbivores to sequester higher concentrations of cardenolides on AMF-colonized plants due to AMF-mediated increases in the cardenolide concentrations of their host plants. Furthermore, we expected that AMF colonization would improve the performance of aphids and caterpillars by increasing plant nutritive quality and biomass, outweighing the negative effects of increased cardenolide concentrations on the herbivores. However, because the outcomes of many AMF-plant associations are species-specific (above), we again expected that the magnitude of the effects of AMF on herbivore sequestration and performance would vary among plant species and with the level of AMF inoculum available to the plant.

Chapter IV. Mycorrhizae alter constitutive and herbivore-induced volatile emissions by milkweeds

In addition to influencing plant direct defenses and plant nutritive quality (Chapters II and III), AMF may also shape multitrophic interactions by altering plant volatile emissions. Plants use VOCs to cue natural enemies to their herbivore prey on plants, reducing damage by herbivores (Kessler and Baldwin 2001, Turlings and Erb 2018). Simultaneously, herbivores utilize volatile cues to identify appropriate hosts (Bruce et al. 2005, Bruce and Pickett 2011). Despite extensive efforts to understand sources of variation in plant communication by VOCs (Turlings and Erb 2018), we still lack an understanding of how ubiquitous mutualists of plants, such as AMF, influence plant constitutive and herbivore-induced VOC emissions. Therefore, in this chapter I evaluate how AMF affect plant constitutive and herbivore-induced VOC emissions.

We performed a full-factorial experiment, manipulating oleander aphids (*Aphis nerii*) on two milkweed species (*Asclepias incarnata* and *A. curassavica*) provided with zero, medium, or high amounts of AMF inoculum. Based on previous studies of *A. nerii* (Zehnder and Hunter 2007; de Roode et al. 2011), we expected aphid feeding to decrease plant direct and indirect defenses to varying extents between plant species. Furthermore, we expected AMF to alter both constitutive and aphid-induced VOC emissions in a plant species-specific manner, with the strength of these effects varying with AMF availability. Because the outcomes of AMF-plant associations on VOC emissions are specific to the AMF and plant species (Rapparini et al. 2008, Fontana et al. 2009, Leitner et al. 2010, Asensio et al. 2012, Schausberger et al. 2012, Babikova et al. 2014b, 2014a, Shrivastava et al. 2015), we did not have specific predictions for the direction of these effects.

Chapter V. **Arbuscular mycorrhizal fungi alter herbivore-predator interactions**

In Chapters II-IV, I demonstrate that AMF may affect multitrophic interactions from the bottom-up by altering plant quality for herbivores (Hartley and Gange 2009, Koricheva et al. 2009). In addition, I show that AMF may also affect multitrophic interactions from the top-down by affecting herbivore resistance to their natural enemies and altering herbivore-induced plant volatile emissions (Rapparini et al. 2008, Fontana et al. 2009, Leitner et al. 2010, Asensio

et al. 2012, Schausberger et al. 2012, Babikova et al. 2014b, 2014a, Shrivastava et al. 2015). These findings suggest that AMF may have pervasive effects on interactions among plants, herbivores, and their natural enemies. Therefore, in Chapter V, I performed a field experiment to evaluate the ecological relevance of AMF on multitrophic interactions. We hypothesized that AMF would increase colonization by both herbivores and their natural enemies. We did not have specific predictions for the effects of AMF on herbivore abundance, as the effects of AMF on plant phenotype and natural enemies may combine to shape herbivore abundances. Lastly, because the outcomes of AMF-plant associations are specific to the AMF and plant species involved (above) we again expected the magnitude of these effects to vary with AMF availability differentially among milkweed species.

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Chapter II

Arbuscular mycorrhizal fungi mediate herbivore-induction of plant defenses differently above and belowground

Abstract

Plants are exposed to herbivores and symbionts above and belowground. Herbivores aboveground alter plant defenses in both leaves and roots, affecting plant-herbivore interactions above and belowground. Root symbionts, such as arbuscular mycorrhizal fungi (AMF), also influence the defenses of leaves and roots, and alter plant responses to herbivory. However, we lack an understanding of how AMF mediate plant responses to herbivores simultaneously in above and belowground plant tissues, despite the ubiquity of such interactions and their consequences for ecological communities. In a full factorial experiment, we subjected plants of four milkweed (*Asclepias*) species under three levels of AMF inoculum availability to damage by aphids (*Aphis nerii*), caterpillars (*Danaus plexippus*), or no herbivores. We then measured foliar and root cardenolides (chemical defenses), leaf toughness, latex exudation (physical defenses), foliar carbon, nitrogen, and phosphorous concentrations, plant biomass, and levels of AMF colonization of roots.

Plants inoculated with AMF generally produced tougher leaves with higher cardenolide concentrations than did plants without AMF. In contrast, root cardenolides were altered by AMF inoculum availability in a plant species-specific manner. The relative induction or suppression of foliar cardenolides and leaf toughness by herbivores was altered strongly by the level of AMF inoculum available to plants. However, AMF did not influence caterpillar-induction or aphid-suppression of root cardenolides. In addition, herbivore feeding induced substantial changes in levels of AMF colonization of roots in a plant species-specific manner. We demonstrate that the availability of AMF in soil alters herbivore induction and suppression of plant defenses strongly, and does so differently in above and belowground plant tissues. Furthermore, we show that

herbivore feeding alters levels of AMF colonization substantially, completing a feedback loop between above and belowground organisms. Our study suggests that indirect interactions between AMF and herbivores may have community-wide consequences by altering plant phenotype both above and belowground.

Introduction

Although spatially separated, organisms aboveground have substantial impacts on belowground organisms, and *vice versa*. These interactions often take place indirectly through plants, which have organs above and below the soil surface that link organisms above and below ground (van der Putten et al. 2001, Bezemer and van Dam 2005, Erb et al. 2008, Pineda et al. 2010, van Dam and Heil 2011, Johnson et al. 2012, Stam et al. 2014). For example, herbivores feeding on leaf tissue alter the performance of root herbivores and the composition of microbial communities in soil and roots (Hol et al. 2004, Kaplan et al. 2008, Bezemer et al. 2013). Similarly, root feeders and soil microbes impact the performance of aboveground insect herbivores (Erb et al. 2008, Koricheva et al. 2009, Pineda et al. 2010, Soler et al. 2013a, Rasmann et al. 2017). Although ubiquitous, the mechanisms and ecological consequences of such interactions are just beginning to be understood (Erb et al. 2009, Papadopoulou and van Dam 2017, De Deyn 2017).

Indirect interactions between above and belowground organisms are generally mediated by changes in plant phenotype. In response to herbivore damage, plants often increase physical and chemical defenses, and decrease their nutritive quality, rendering plants less palatable to future herbivores (Karban and Baldwin 1997, Karban 2011, Barton 2016). Plant responses to herbivory are not restricted to locally damaged organs, and can be expressed in undamaged tissues (Erb et al. 2008). For example, shoot herbivory in *Senecio jacobaea* decreases concentrations of pyrrolizidine alkaloids in roots, although it does not affect alkaloid levels in shoots (Hol et al. 2004). In addition, plant responses to herbivory vary with the feeding mode of the herbivore because herbivore guilds induce distinct defensive signaling pathways in plants (Kessler and Baldwin 2002, Erb et al. 2012). For instance, chewing herbivores, such as caterpillars, interact primarily with the jasmonic acid (JA) pathway, while phloem-feeding insects, such as aphids, interact with the salicylic acid (SA) pathway (Kessler and Baldwin

2002). As a consequence, caterpillar feeding often induces plant defenses, reducing the performance of future herbivores (Ali and Agrawal 2014). In contrast, the responses of plants to aphid feeding are far more variable; aphids often manipulate plant quality to their own benefit (Züst and Agrawal 2016) and to the benefit of other herbivores (Ali and Agrawal 2014).

Plant responses to herbivore feeding are also shaped by interactions with belowground microbial symbionts, such as arbuscular mycorrhizal fungi (AMF) (Bennett et al. 2009, Kempel et al. 2010, Barber 2013, Babikova et al. 2014, Wang et al. 2015, Minton et al. 2016, He et al. 2017). AMF colonize the roots of over 80% of plant species globally and provide nutrients and water to plants in exchange for sugars (Wang and Qiu 2006, Smith and Read 2008, Soudzilovskaia et al. 2015). AMF interact with plant defensive signaling pathways, generally upregulating the JA pathway while suppressing the SA pathway, in addition to interacting with various other plant hormones (Jung et al. 2012, Cameron et al. 2013, Gutjahr 2014, Bucher et al. 2014). By altering plant nutrient uptake and interacting with plant defensive signaling pathways, AMF alter plant nutritive quality and resistance traits, and prime plants to defend against attack, leading to greater and more rapid expression of defense genes after damage (Jung et al. 2012, Song et al. 2013, He et al. 2017). The association with AMF is often mutualistic for plants, as AMF frequently stimulate plant growth and mitigate abiotic and pathogen stress (Smith and Read 2008). However, the effects of AMF on plant growth and defense range from beneficial to detrimental, depending on the environment (Hoeksema et al. 2010), plant and AMF identity (Klironomos 2003, Tao et al. 2016), and the density of AMF inoculum available to the plant (Garrido et al. 2010, Vannette and Hunter 2011, 2013). The density of AMF inoculum in soils, measured as infectivity and spore abundances, varies on small scales, such as centimeters (Wolfe et al. 2007) and meters (Carvalho et al. 2003). Therefore, plants within a single population may experience substantial variation in AMF availability. However, no study to date has considered how this variation in AMF availability may affect plant responses to herbivore feeding.

Effects of AMF on the induction of chemical defenses by herbivores aboveground appear highly variable among systems. For example, several studies suggest that plants can only induce defenses in response to herbivore feeding when colonized by AMF (Kempel et al. 2010, Barber 2013). In contrast, other studies report that, while AMF increase constitutive levels of defenses,

AMF actually suppress the induction of defenses by herbivores (Bennett et al. 2009, Wang et al. 2015). Thus far, studies linking herbivore-induction of direct defenses aboveground with AMF colonization have been limited to chewing (lepidopteran) herbivores, although AMF can influence aphid-induction of plant indirect defenses substantially (i.e. volatile organic compound emissions; Guerrieri et al. 2004, Babikova et al. 2014).

In contrast to these studies linking AMF colonization to herbivore-induced defenses aboveground, we are unaware of any study to date that examines how AMF may mediate the response of plant roots to aboveground herbivory. This is despite increasing recognition of the importance of aboveground herbivory in mediating belowground plant-herbivore interactions (Erb et al. 2008, Johnson et al. 2012, Huang et al. 2014, Mundim et al. 2017). Moreover, shoot-feeding herbivores aboveground influence AMF colonization of plant roots (Gehring and Bennett 2009, Barto and Rillig 2010), thereby completing an important feedback loop between above- and belowground organisms (Hunter 2016). This may occur through induced changes in carbon allocation in plants (Gehring and Whitham 2002, Gehring and Bennett 2009) or altered phytohormonal signaling (Landgraf et al. 2012, Fragoso et al. 2014, He et al. 2017). Because AMF colonization affects the performance of herbivores (Koricheva et al. 2009), and AMF perform important ecosystem services, such as soil aggregation, nutrient cycling, and carbon sequestration (Smith and Read 2008), herbivore-induced changes in levels of AMF colonization could have community and ecosystem-level consequences. Most studies to date have considered only how defoliating herbivores affect AMF colonization of roots (Gehring and Bennett 2009, Barto and Rillig 2010, but see Babikova et al. 2014, Vannette and Hunter 2014). However, because herbivores of different feeding guilds alter plant allocation of resources differentially (Kaplan et al. 2008, 2011, Tao and Hunter 2013), they could affect AMF colonization differently.

Here, we address how AMF mediate herbivore induction of plant defenses above and belowground, and how herbivore feeding affects AMF colonization. We performed a full factorial experiment, manipulating two specialist herbivores (one chewing and one phloem-feeding) on four closely related plant species provided with different amounts of AMF inoculum. Specifically, we asked: (1) How do AMF mediate the induction of foliar defenses by different

aboveground herbivores? (2) How do AMF mediate the response of root defenses to aboveground herbivory? (3) How is AMF colonization affected by different aboveground herbivores? We predicted that (1) caterpillar feeding (chewing) would increase plant defenses to varying extents among plant species, whereas aphid feeding (phloem-feeding) would suppress plant defenses. We expected AMF to enhance plant responses to caterpillars, but attenuate plant responses to aphids. Furthermore, we expected the strength of these effects to vary with AMF availability. (2) We had no specific prediction for effects of AMF on the induction of root defenses by aboveground herbivores because responses of root traits to shoot herbivory are highly variable among plant and herbivore species (Erb et al. 2008). (3) We predicted that aphid feeding would decrease mycorrhizal colonization, but short-term caterpillar feeding would increase mycorrhizal colonization. However, because the outcomes of many AMF-plant associations are specific to the AMF and plant species (e.g. Grman 2012, Barber et al. 2013, Anacker et al. 2014, Tao et al. 2016), we expected the strength of these effects to vary among plant species.

Materials and methods

Study System

We used milkweed (*Asclepias*) species, to investigate how AMF colonization of roots influences constitutive and herbivore-induced plant resistance traits in foliar and root tissues. Milkweeds provide an ideal system in which to address these questions because milkweeds produce a suite of resistance traits in leaves and roots that affect milkweed herbivores substantially, despite herbivore adaptations to resist the defenses of milkweeds (Agrawal 2004, Rasmann et al. 2009a, Rasmann and Agrawal 2011). In their foliar and root tissues, milkweeds produce cardenolides, bitter tasting steroids that disrupt the functioning of sodium-potassium channels in animal cells (Agrawal et al. 2012b). Depending on herbivore identity, herbivore feeding can increase or decrease cardenolide concentrations in leaves (Zehnder and Hunter 2007, de Roode et al. 2011, Agrawal et al. 2014) and in roots (Rasmann et al. 2009a, 2011, Erwin et al. 2014). In response to leaf damage, milkweeds exude latex, a sticky isoprene polymer that gums up the mouths of chewing herbivores (Zalucki et al. 2001, Agrawal and Konno 2009); latex exudation can also increase in response to previous herbivory (Agrawal et al. 2014). In addition,

milkweed species vary in leaf toughness (Agrawal and Fishbein 2006), which is tightly correlated with specific leaf mass (SLM) (Frost and Hunter 2008).

We used four North American milkweed species, *Asclepias curassavica, A. latifolia, A. syriaca*, and *A. incarnata*, that show constitutive and AMF-mediated variation in foliar and root cardenolide concentrations (Vannette et al. 2013, Tao et al. 2016). *Asclepias incarnata* and *A. syriaca* seeds were collected from naturally occurring populations in Livingston County, MI, and *A. latifolia* and *A. curassavica* seeds were purchased from commercial sources (Alplains and Butterfly Encounters Inc., respectively). We obtained fungal inoculum from Mycorrhizal Applications (Grants Pass, OR, USA), which was comprised of cosmopolitan AMF species, including *Rhizophagus intraradices*, *Funneliformis mosseae*, *Glomus aggregatum*, and *Claroideoglomus etunicatum* (33 spores of each AMF species per gram of inoculum, www.plant-success.com). However, cloning and Sanger sequencing of the inoculum revealed that the inoculum consisted only of *Funneliformis mosseae* (Meier and Hunter 2018). Milkweeds grow in habitats that host a diversity of AMF taxa (Öpik et al. 2006), and can form associations with these cosmopolitan AMF species in natural and experimental populations (Landis et al. 2004, Vannette and Hunter 2011, Vannette et al. 2013, Tao et al. 2016). However, as with most systems, the frequency of these relationships is not known.

Experimental protocols

We surface-sterilized seeds in 5% bleach and germinated them at room temperature after six weeks of cold, moist stratification at 4 °C (the tropical *A. curassavica* did not require stratification). Individual seedlings were planted in conical deepots (Steuwe and Sons Inc., Corvalis, OR, USA) with depth of 25 cm and diameter of 6.4 cm, filled with 600 ml autoclaved potting soil (Metro-Mix 380; MetroMix Sun Gro Horticulture Canada CM Ltd., Vancouver, BC, Canada) and sand (3:1 soil:sand) containing AMF inoculum. We manipulated the amount live and autoclaved (dead) AMF inoculum available to experimental plants to generate zero, medium, and high levels of root colonization, which is possible because the amount of AMF inoculum available to milkweed plants influences the extent to which roots are colonized (Vannette and Hunter 2011, 2013, Tao et al. 2016). Specifically, we homogenized 4.20 g autoclaved AMF inoculum (zero treatment), 1.20 g live and 3.00 g autoclaved inoculum (medium treatment), or

4.20 g live inoculum (high treatment) in 200 ml of autoclaved soil and sand. This fungal-soil mixture was placed atop 200 ml autoclaved soil, and covered by 200 ml autoclaved soil to prevent transfer of mycorrhizal spores or hyphae among treatments. We restored the natural bacterial community to the soil of each pot by adding 20 ml of bacterial solution that was made by suspending 100 ml of potting soil in 1 L deionized water and filtering the suspension through an ultra-fine soil sieve (38 μm) to exclude AMF hyphae and spores. Plants were grown for three months at the Matthaei Botanical Gardens greenhouses (Ann Arbor, MI) with a photoperiod of 16:8 L:D. Plants were watered *ad libitum* and fertilized biweekly with 90 ml of a low concentration (94 ppm) of 15-0-15 (N-P-K) dark weather fertilizer (JR Peters Inc., Allentown, PA). All experimental plants were exposed to colonization and damage by greenhouse thrips; plants were sprayed monthly with a mixture of Enstar, Lucid, and MPede to minimize damage. No pesticides were sprayed for three weeks prior to the addition of herbivores; thrips were killed weekly by hand during this period.

To assess the effects of AMF on the induction of plant resistance traits above and belowground by herbivores of different feeding guilds, we used two specialist herbivores: oleander aphids (phloem-feeding; *Aphis nerii*) and monarch caterpillars (leaf-chewing; *Danaus plexippus*). All oleander aphids used in the experiment were clones derived from a single aphid collected in March 2014 from the Emory University greenhouses (Atlanta, GA) and reared indoors on *A. tuberosa*. Monarch larvae were the outcrossed progeny of butterflies obtained from Shady Oak Farms (www.butterfliesetc.com), Mr. Butterfly (www.mrbutterflies.com), and Butterfly Release Company (www.butterflyreleasecompany.com), and raised in a growth room with photoperiod of 16:8 L:D on a combination of *A. syriaca*, *A. incarnata*, and *A. curassavica*.

In a fully factorial design, we subjected plants of each species and AMF treatment to either damage by oleander aphids, monarch caterpillars, or no herbivores (control treatment). All plants were covered with white, nylon mesh bags to prevent insect movement among experimental plants. Five reproductive, apterous oleander aphids were placed at the apex of 15 replicates of each plant species x AMF treatment and allowed to reproduce naturally for six days (n=180). Dead or missing reproductive aphids were replaced on the second day. One newly hatched monarch caterpillar was placed on each of 20 replicates of each plant species x AMF

treatment and allowed to feed for six days (n=240). Missing or dead caterpillars were replaced on the second day. 20 plants of each plant species x AMF treatment experienced no herbivory but were covered with white, nylon mesh bags to control for effects of mesh on plant traits (n=240). We conducted this experiment in four temporal blocks separated by one day, with each treatment equally represented in each temporal block.

After the six days of feeding, herbivores were removed and plants were harvested destructively to measure plant resistance and nutritive traits, biomass, and AMF colonization of roots. Leaves damaged by caterpillars were removed, scanned, and the area consumed determined with Image J (Schneider et al. 2012).

Analysis of plant traits

To measure foliar traits, we punched three fresh leaf disks from each leaf of the sixth leaf pair (six hole punches, 424 mm^2 total) of each plant, placed the disks in 1 mL of methanol, and stored them at -10 °C until cardenolide analysis. Latex that exuded from the hole punches was collected on pre-weighed cellulose disks, dried at 50 °C, and weighed. Six additional leaf disks were taken from the same leaves, stored in glassine envelopes, dried at 50 °C, and weighed to estimate SLM and dry mass of foliar material used in cardenolide analyses. SLM was estimated by dividing mass of dried leaf disks by total disk area as a proxy for leaf toughness (Frost and Hunter 2008). Additional leaves from neighboring leaf pairs were removed and dried at 50 °C for subsequent carbon, nitrogen, and phosphorus analyses. Remaining plant material was dried at 50 °C in paper bags and weighed to measure aboveground biomass after correcting for foliar tissue removed by caterpillars or chemistry sampling.

Only a subset of roots of all experimental treatments were analyzed (10 replicates of each AMF x plant species x herbivore treatment, n=360) due to time constraints in harvesting. After washing the roots carefully in deionized water, we sub-sampled them as follows: a) we stored 150 mg of 1 cm pieces of fresh fine root tissue in 60% ethanol at 4 °C until we could quantify AMF colonization; b) we stored 50 mg of fresh fine root tissue in 1 ml of methanol at -10 °C for subsequent cardenolide analysis; c) we weighed approximately 400 mg of fresh fine root, dried it at 50 °C, and reweighed samples to calculate wet weight/dry weight ratios from which to

estimate the dry mass of the subsamples in a) and b), above. We dried all remaining root tissue at 50 °C and weighed its contribution to total root biomass.

Foliar and root cardenolide concentrations were assessed following well-established methods (Zehnder and Hunter 2007). Leaf disks and fine root samples were ground for 3 min in methanol, sonicated for 1 h, and then centrifuged for 6 min. The supernatant was evaporated under vacuum at 45 °C until dry and resuspended in 150 µl methanol containing 0.15 mg ml^{-1} digitoxin as an internal standard. Samples were then separated by ultra performance liquid chromatography (UPLC; Waters Inc, Milford, MA, USA) using a Luna 2.5 µm C18(2) column (50 x 2 mm, Phenomenex Inc., Torrance, CA, USA). Each 2 µl injection was eluted at a constant flow of 0.7 ml per min with a gradient of acetonitrile and water for the 9 min run, maintaining at 20% acetonitrile for 3 min, and increasing to 45% acetonitrile for 5 min, and then maintaining at 20% acetonitrile for 1 min. Peaks were detected by a diode array detector at 218 nm, and absorbance spectra recorded from 200 to 400 nm. Symmetric peaks with maximum absorbance between 217 - 222 nm were quantified as cardenolides. Cardenolide concentrations were calculated using the digitoxin internal standard and total cardenolide concentrations were calculated as the sum of individual peaks.

Carbon (C) and nitrogen (N) concentrations of foliar tissues were measured with a TruMac elemental analyzer (Leco Corporation, St. Joseph, MI, 49085, USA). Phosphorous (P) concentrations of foliar samples were determined by dry combusting ground samples in a muffle furnace at 550 °C overnight, followed by persulfate digestion at 121 °C for 60 min in an autoclave, and analysis by the molybdenum blue method on a PowerWave XS plate reader (Bio-Tek, Highland Park, Winooski, Vermont, 05404, USA) reading at 880 nm. P concentrations of samples were calculated from a potassium phosphate standard curve and quality control was assessed with NIST apple leaf standard analyzed with all samples. Only a subset of all experimental treatments were analyzed for nutritive traits, due to time and financial constraints (10 replicates of each plant species x AMF x herbivore treatment, n=360).

To quantify AMF colonization, roots were cleared with 10% KOH for 10 min, acidified using 2% HCl, and stained in 0.05% trypan blue in 1:1:1 water:glycerol:lactic acid (Vannette and

Hunter 2011). We mounted stained roots on slides and scored AMF colonization using the magnified gridline intersect method (McGonigle et al. 1990) with a Nikon compound microscope (Melville, NY, USA). A root intersection was considered colonized if hyphae, arbuscules, or vesicles were present. At least 100 root intersections were analyzed per plant.

Data analyses

For both leaves and roots of each sample, we calculated three measures of cardenolide expression, including total cardenolide concentration (sum of all cardenolide peaks), cardenolide diversity (using Shannon's index), and cardenolide polarity (relative representation of lipophilic cardenolides), by summing the relative peak areas multiplied by each peaks' retention time (Rasmann and Agrawal 2011, Sternberg et al. 2012). Evidence suggests that a greater diversity of cardenolides and more lipophilic cardenolides are more toxic than are lower diversity or more polar mixes (Fordyce and Malcolm 2000, Zehnder and Hunter 2007, Sternberg et al. 2012). We also evaluated differences in foliar and root cardenolide composition (i.e. identity and relative abundance) among plant species, AMF treatment, herbivore damage, and their interactions using permutational multivariate ANOVA (PERMANOVA; McCune et al. 2002). We used the adonis function in the vegan package (Oksanen et al. 2016) in R v 3.3.1 and calculated dissimilarities among samples using the Bray-Curtis metric for PERMANOVA.

To compare the effects of AMF inoculum availability and herbivore damage on plant traits and levels of AMF colonization among milkweed species, we used general linear mixed models. Milkweed species, AMF inoculum availability, herbivore damage, and their interactions were fixed factors and each plant trait was a dependent variable. In all analyses, the temporal block of the experiment was designated as a random effect. We fitted models for each plant trait separately. The residuals of all analyses were checked for normality and homogeneity of variance. Data were natural log-transformed when necessary. All statistical analyses were performed in SAS 9.4 (SAS Institute, Cary, NC, USA). Sample sizes were initially 15-20 plants per treatment for foliar defensive traits and aboveground biomasses, 10 plants per treatment for foliar nutritive traits, and 10 plants per treatment for root defensive traits and belowground biomasses. However, samples sizes ranged from 12-20 plants per treatment for foliar defensive

traits and aboveground biomasses, 8-10 for foliar nutritive traits, and 8-10 for root defensive traits and belowground biomasses due to samples lost during processing or chemical analyses.

Results

We summarize the effects of milkweed species, AMF inoculum availability, herbivore feeding, and their interactions, on all plant traits in Table 2.1. We describe key results in more detail below. Note that we have reported the effects of AMF on the traits of the undamaged (control) milkweeds previously in a manuscript on herbivore performance (Meier and Hunter 2018). Here, we use data from the control plants only to measure the magnitude of induction in treatment (herbivore-damaged) plants.

Mycorrhizal colonization

Inoculation of plants with AMF resulted in successful root colonization, whereas control plants remained AMF-free (AMF $F_{2,315}$=99.81; P<0.0001; Fig. A2.1). The proportion of roots colonized by arbuscules was tightly correlated with colonization by all fungal structures (R^2=0.96, P<0.0001), so we report only the latter. Levels of AMF colonization of roots were not a simple function of inoculum availability; analysis of plants inoculated with live AMF (medium and high AMF treatments only) revealed that while AMF colonization varied among plant species (Plant species $F_{3,210}$=7.41, P<0.0001; Fig. A2.1), medium and high AMF treatments resulted in equivalent levels of AMF colonization (AMF $F_{1,210}$=1.12 P=0.2912; Plant species*AMF $F_{3,210}$=0.87, P=0.457; Fig. A2.1). However, herbivore induction of plant defenses varied substantially between medium and high AMF treatments (below), so the availability of inoculum must have had effects on plant phenotype beyond those observed by estimates of colonization alone. We have therefore continued to treat medium and high AMF treatments separately in all following analyses.

Effects of AMF on plant defenses aboveground

Inoculation with AMF increased foliar cardenolide concentrations in the three milkweed species that expressed cardenolides aboveground (AMF $F_{2,449}$=9.45, P<0.0001; Fig. 2.1a). *Asclepias incarnata* produced no foliar cardenolides in this study, and was therefore excluded from foliar cardenolide analyses. Foliar cardenolide diversity was greatest in milkweed plants

under medium AMF inoculum availability, although the magnitude of increase was largest in *A. syriaca* (Plant species*AMF $F_{4,436}$=3.1, P=0.0156; Fig. 2.1b). AMF inoculum availability also shifted the composition of foliar cardenolides in a plant species-specific manner (PERMANOVA Plant species*AMF $F_{4,438}$=3.67, P<0.001). In addition, AMF inoculation generally increased leaf toughness (SLM), especially under medium AMF availability, in *A. curassavica*, *A. incarnata*, and *A. syriaca* (Plant species*AMF $F_{6,608}$=4.97, P<0.0001, Fig. 2.1c). Latex exudation was unaffected by AMF inoculum availability (Table 2.1).

Notably, rather than inducing increases in foliar cardenolide concentrations, it was more common for herbivores to suppress cardenolide concentrations in milkweed leaves (Figs. 2.2a,b). However, the degree of cardenolide suppression varied among herbivores, milkweed species, and AMF inoculum availability (three-way interaction $F_{8,449}$=2.22, P=0.0251, Figs. 2.2a,b). The three-way interaction is most clearly illustrated by plotting effect sizes as Hedge's D, which show the influence of herbivores on cardenolide concentrations relative to concentrations in control plants. Aphids suppressed foliar cardenolide concentrations under high levels of AMF availability by 31% in *A. curassavica* and by 62% in *A. syriaca* (Fig. 2.2a). Caterpillar feeding suppressed foliar cardenolide concentrations under medium and high AMF treatments in *A. latifolia* by 32% and 40%, respectively (Fig. 2.2b).

Similarly, herbivore feeding often, though not uniformly, suppressed foliar cardenolide diversity (Figs. 2.2c,d). Overall, herbivore effects on cardenolide diversity varied in a manner specific to the plant species and AMF treatment (Plant species*Herbivore $F_{4,436}$=4.84 P=0.0008; AMF*Herbivore $F_{4,436}$=3.84, P=0.0045; Figs. 2.2c,d). For example, aphid feeding increased cardenolide diversity by 18% in the leaves of *A. latifolia* under medium AMF, but decreased cardenolide diversity in the leaves of *A. syriaca* by 69% under high AMF (Fig. 2.2c). Caterpillar feeding decreased foliar cardenolide diversity in *A. syriaca* by 66% under both zero and high AMF availability, but did not affect cardenolide diversity in any other plant species (Fig. 2.2d). There were also relatively minor, but significant, species-specific effects of herbivore feeding on the composition of cardenolides in leaves (PERMANOVA Plant species*Herbivore $F_{4,438}$=3.12, P<0.001).

Unlike the general pattern of cardenolide suppression by herbivores noted above, herbivores could either induce or suppress the toughness (SLM) of milkweed leaves, with AMF inoculation enhancing induction and attenuating suppression of toughness (Three-way interaction $F_{12,608}$=1.91, P=0.0303, Figs. 2.3a,b). For example, aphid feeding increased leaf toughness in *A. curassavica*, *A. incarnata*, and *A. syriaca* by 13%, 22%, and 25%, respectively, under medium AMF inoculum availability (Fig. 2.3a). Aphid feeding suppressed leaf toughness in *A. latifolia* by 13% and caterpillar feeding suppressed leaf toughness in *A. incarnata* by 10% in AMF-free plants, but did not suppress leaf toughness in *A. latifolia* or *A. incarnata* plants colonized by AMF (Figs. 2.3a,b). Regardless of AMF treatment, aphid and caterpillar feeding suppressed latex exudation in *A. latifolia* by 24% and 27%, respectively, but did not affect latex exudation in any other plant species (Plant species*Herbivore $F_{6,598}$=3.14, P=0.0049).

Effects of AMF on plant defenses belowground

In contrast to AMF-mediated increases in foliar cardenolide concentrations (above), root cardenolide concentrations were unaffected by AMF inoculum availability (AMF $F_{2,304}$=0.64, P=0.5303, Table 2.1). However, AMF had substantial, plant species-specific effects on root cardenolide diversity (Plant species*AMF $F_{6,304}$=4.54, P=0.0002, Fig. 2.4) and the composition of cardenolide types in roots (PERMANOVA Plant species*AMF $F_{6,306}$=2.41, P=0.002). AMF inoculation increased cardenolide diversity in the roots of *A. curassavica* and *A. incarnata*, but reduced cardenolide diversity in the roots of *A. latifolia* and *A. syriaca* (Fig. 2.4).

In comparison to the complex interactions among plant species, AMF inoculum availability, and herbivore damage on the induction and suppression of foliar defenses (above), effects of herbivores on cardenolide expression belowground were simple; aphids suppressed root cardenolide concentrations across all milkweed species and AMF treatments by an average of 10%, while caterpillar feeding increased root cardenolide concentrations by an average of 5% (Herbivore $F_{2,304}$=4.86, P=0.0084, Fig. 2.5). Herbivores shifted, slightly, the composition of cardenolides types in roots differentially among plant species and AMF treatments (PERMANOVA Three-way interaction $F_{12,306}$=1.56, P=0.022), had minor effects on root cardenolide polarity, and no influence on root cardenolide diversity (Table 2.1).

Effects of herbivores on AMF colonization of roots

Critically, both aphids and caterpillars had substantial impacts on root colonization by AMF, although herbivores fed on experimental plants for only six days (Fig. 2.6). This highlights that even short-term herbivore activity can influence markedly the interactions between plants and their AMF. The effects of herbivores on levels of AMF colonization varied in both magnitude and direction among milkweed species (Plant species*Herbivore $F_{6,210} = 2.54$, P=0.0216, Fig. 2.6). For example, aphid feeding increased AMF colonization of *A. incarnata* roots by 56%, while decreasing colonization of *A. curassavica* and *A. syriaca* roots by 36% and 25%, respectively. Likewise, caterpillar feeding increased AMF colonization of *A. latifolia* roots by 38%, while decreasing colonization of *A. syriaca* roots by 43% (Fig. 2.6).

Effects of AMF and herbivores on plant nutritive and growth traits

Inoculation with AMF increased foliar C/N ratios by an average of 15% (AMF $F_{2,316}$=6.05, P=0.0026; Fig. 2.7a) and reduced foliar N concentrations by an average of 10% (AMF $F_{2,316}$=6.61, P=0.0015; Fig. 2.7b) under medium and high AMF availability. However, AMF influenced foliar P concentrations differentially among plant species (Plant species*AMF interaction $F_{6,315}$=6.76, P<0.0001; Fig. 2.7c). AMF inoculation increased foliar P concentrations by an average of 7% in *A. curassavica* and 22% in *A. latifolia*, but reduced foliar P concentrations by an average of 20% in *A. incarnata* and 12% in *A. syriaca* (Fig. 2.7c). Foliar C concentrations were unaffected by AMF inoculum availability (Table 2.1).

AMF inoculum availability had no effect on the relative allocation of plant biomass above and belowground (i.e. root/shoot biomass ratio, Table 2.1). However, whether plant above and belowground biomass increased or decreased when inoculated with AMF varied among milkweed species (Plant species*AMF aboveground $F_{6,596}$=5.41, P<0.0001, Fig. 2.7d; belowground $F_{6,315}$=5.14, P<0.0001, Fig. 2.7e). For example, both above and belowground biomass of *A. curassavica* increased when inoculated with AMF, whereas AMF inoculation generally reduced above and belowground biomass of *A. incarnata*, *A. latifolia*, and *A. syriaca* (Figs. 2.7d,e).

Aphids and caterpillars had opposing, albeit minor, effects on foliar nutrient concentrations. Across all AMF treatments and plant species, aphids increased foliar nutrient levels while caterpillar feeding decreased them (Herbivore main effect on C concentration $F_{2,316}$=10.46, P<0.0001; N concentration $F_{2,316}$=3.78, P=0.0239; P concentration $F_{2,315}$=6.48, P=0.0017). Aphid feeding increased foliar C concentrations by 2%, N concentrations by 8%, and P concentrations by 4%, while caterpillar feeding decreased foliar C concentrations by 1%, N concentrations by 2%, and P concentrations by 6%. Caterpillar feeding also decreased the allocation of plant biomass to roots relative to shoots by 14%, but aphid feeding did not affect the relative allocation of plant biomass (Herbivore $F_{2,307}$=6.93, P=0.0011). Herbivore feeding did not affect foliar C/N ratios, plant aboveground biomass, or belowground biomass (Table 2.1).

Discussion

Our study is among the first to document the impacts of AMF on constitutive and herbivore-induced defenses simultaneously in above and belowground tissues. We demonstrate that (1) milkweeds colonized by AMF generally produce tougher and more toxic leaves than do AMF-free plants. Furthermore, the relative induction or suppression of foliar defenses by aphids and caterpillars is altered by the availability of AMF inoculum. (2) AMF inoculum availability induces plant species-specific changes in the chemical defenses of roots, but does not influence the suppression (by aphids) or induction (by caterpillars) of root chemical defenses. (3) Finally, herbivore feeding leads to substantial changes in levels of AMF colonization of roots after just a few days, with the magnitude and direction of those changes varying with plant and herbivore species. This last result is important because it completes a fundamental feedback loop from populations of microbial root mutualists belowground, through alterations in plant phenotype aboveground, to changes in the effects of herbivore feeding on plant traits, and back down to the root mutualists (Hunter 2016).

We observed that differing levels of AMF inoculum availability resulted in differences in the magnitude and direction of herbivore-induced defenses, despite resulting in similar levels of root colonization. Plants maintain a maximum level of AMF colonization and suppress further colonization (Vierheilig et al. 2000a, 2000b, Vierheilig 2004, Meixner et al. 2005) by regulating phytohormones, including JA and SA (Staehelin et al. 2011, Gutjahr 2014, Bucher et al. 2014,

and references therein). These hormones play key roles in the regulation of resistance responses of plants to insect herbivores (Pieterse et al. 2012, 2014). Stronger regulation of AMF colonization by plants through changes in phytohormones in our high than medium AMF treatments may have led to the different responses of plants to herbivory between the AMF treatments. Alternatively, the different effects of medium and high AMF availability on plant phenotype may have resulted from differential colonization by the four AMF species under medium and high AMF availability. The commercial AMF mix was purported to consist of four AMF species, and AMF species vary in nutrient trading and resulting effects on plant phenotype (Bennett et al. 2009, Thonar et al. 2014, Argüello et al. 2016). However, we consider this explanation to be unlikely; cloning and Sanger sequencing of the AMF mix, and of milkweed roots from plants grown under the same experimental conditions, revealed that the AMF mix consisted only of *Funneliformis mosseae* (Meier and Hunter 2018). Therefore, the differential effects of medium and high AMF inoculum availability on herbivore-induction of defenses are more likely due to plant suppression of AMF colonization under high AMF inoculum availability than to differential colonization by AMF species.

Effects of AMF and herbivores on aboveground defenses

The general increase in foliar cardenolides mediated by AMF was not a simple consequence of improved plant nutrition; there were no clear associations between cardenolide expression and either foliar nutrient concentrations or plant biomass. Instead, AMF inoculation may have increased defense metabolites through interactions with phytohormonal signaling pathways (García-Garrido et al. 2002, Jung et al. 2012, Song et al. 2013, He et al. 2017). The induction of cardenolides in milkweeds is related to bursts in JA concentrations (Agrawal et al. 2014). Therefore, AMF-mediated increases in cardenolide concentrations in milkweed plants may result from systemic upregulation of JA, not altered nutrient availability. Similarly, *Plantago lanceolata* and *Nicotiana tabacum* plants exhibit higher levels of foliar defense metabolites in response to AMF colonization, which do not correlate with nutrient availability (Bennett et al. 2009, Andrade et al. 2013).

In contrast to our finding that AMF mediated increases in foliar cardenolide concentrations, previous studies have found no (Tao et al. 2016) or negative effects of AMF on

foliar cardenolide concentrations (Vannette et al. 2013) in the same milkweed species. In these previous studies, plants were younger (Tao et al. 2016) or colonized by a different community of AMF (Vannette et al. 2013). Effects of AMF on plant traits change over the development of the association (Schweiger et al. 2014, Tomczak and Müller 2017) and AMF species and communities differentially alter plant phenotype (Bennett and Bever 2007, Bennett et al. 2009, Vannette and Hunter 2013). In short, species-specific and ontogenetic variation in both plants and AMF may explain why we observed different responses of foliar cardenolide expression to AMF colonization than those observed in earlier work. In addition, it is possible that our plants were already induced by thrips, whereas plants in previous studies were not. However, we do not believe that the minor thrip damage altered the quality of our results because plants of all treatments were attacked equally. Overall, these differences among studies using the same milkweed species highlight the substantial context-dependent nature of the outcomes of interactions between plants and AMF (Klironomos 2003, Bennett and Bever 2007, 2009, Anacker et al. 2014).

While AMF caused overall increases in leaf defensive chemistry, greater AMF availability in soils also engendered aphid suppression of cardenolides. Aphids may have suppressed foliar cardenolides under high AMF availability simply because aphids reached their highest numbers on milkweed plants under high AMF availability (Table A2.1); aphids decrease foliar cardenolide concentrations in high cardenolide milkweed species in a density-dependent manner (Martel and Malcolm 2004, Zehnder and Hunter 2007). Aphids may have reached sufficient densities on plants under high AMF availability to stimulate cardenolide suppression.

While AMF may increase aphid densities on milkweed, they may also increase the susceptibility of those aphids to natural enemies. Aphids sequester cardenolides in amounts closely correlated with those present in their host plants (Malcolm 1990), and aphids that sequester low concentrations of cardenolides are more susceptible to predation (Malcolm 1992, Mooney et al. 2008). Cardenolide suppression in response to aphid feeding may ultimately benefit milkweeds by increasing aphid susceptibility to predation (Malcolm and Zalucki 1996, Martel and Malcolm 2004, Zehnder and Hunter 2007). Therefore, by mediating the decline in foliar cardenolides in response to aphid feeding, AMF may increase the susceptibility of aphids

to predation, and ultimately decrease aphid-pressure for the plant. Furthermore, AMF colonization alters plant constitutive and aphid-induced volatile organic compounds (Babikova et al. 2014), increasing the attractiveness of host plants to the natural enemies of aphids (Guerrieri et al. 2004, Babikova et al. 2013), and improving natural enemy performance (Bennett et al. 2016). Thus, in addition to altering aphid defenses against predators, AMF may alter plant resistance to aphids indirectly by making plants more attractive to natural enemies and improving natural enemy performance.

In a breadth of plant species, caterpillars only induce increases in plant chemical defense when those plants are colonized by AMF (Kempel et al. 2010, Barber 2013). However, in our study system, AMF colonization either had no effect on caterpillar induction or led plants to reduce cardenolides in response to caterpillar feeding. Similarly, in *Plantago lanceolata*, caterpillar feeding increases foliar defensive chemistry in AMF-free plants, but not in plants colonized by AMF (Bennett et al. 2009, Wang et al. 2015). Effects of AMF availability on caterpillar reduction of cardenolides do not appear to result from altered nutrient availability, but instead may result from interactions between AMF and plant hormonal pathways (Jung et al. 2012, Song et al. 2013, He et al. 2017). Reduction or lack of induction of cardenolides by caterpillars in milkweeds is not common, but can occur depending on the plant species or genotype, duration of caterpillar feeding, and light availability (Rasmann et al. 2009a, Bingham and Agrawal 2010, Agrawal et al. 2012a, Ali and Agrawal 2014). We suggest that high levels of AMF inoculum availability may favor strategies of plant tolerance rather than resistance in these milkweed species, whereby regrowth after damage is enhanced by AMF at the expense of phytochemical induction (Bennett and Bever 2007, Tao et al. 2016, but see Garrido et al. 2010).

As noted above with aphids, AMF also mediated the declines in cardenolide expression caused by monarch caterpillar feeding. Although high concentrations of cardenolides reduce monarch caterpillar performance (Zalucki et al. 2001, Agrawal 2005, Rasmann et al. 2009b), cardenolides also protect monarchs from their predators (Brower et al. 1968, Brower and Moffitt 1974, Malcolm 1994) and pathogens (Lefèvre et al. 2010, Sternberg et al. 2012). Therefore, whether AMF increase or decrease the resistance of milkweeds to subsequent caterpillar attack

will likely depend on trade-offs between direct effects of cardenolides on caterpillar performance and indirect effects on the performance of natural enemies.

In contrast to AMF-mediated declines in cardenolides in response to herbivory, AMF colonization generally enhanced herbivore induction of leaf toughness, especially at medium levels of AMF availability. The effects of AMF on herbivore-induced increases in leaf toughness were associated with the effects of AMF on foliar C/N ratios. Our data show that increases in C/N ratios resulted from declines in foliar N concentrations with no change in foliar C concentrations (Fig. 2.7a,b; Table 2.1). AMF-mediated declines in foliar N concentrations have been reported previously (Bennett et al. 2009, Barber 2013), although the exact mechanism is unclear. However, reductions in mineral nutrient availability may favor the allocation of C to defensive compounds (Herms and Mattson 1992), including C-rich molecules such as cellulose on which leaf toughness depends (Westbrook et al. 2011). Leaf toughness is an effective defense against a diversity of herbivores (Read and Stokes 2006, Clissold et al. 2009, Kos et al. 2011), and thus AMF influence on leaf toughness may protect plants from future damage.

Effects of AMF and herbivores on belowground defenses

As in previous studies (De Deyn et al. 2009, Andrade et al. 2013, Vannette et al. 2013, Bennett et al. 2013, Miller et al. 2014), we found different effects of AMF on foliar and root defenses. Although cardenolides can be synthesized in shoot tissue (Groeneveld et al. 1991), cardenolide biosynthesis may be refined in different plant organs (Manson et al. 2012). AMF likely affect cardenolide biosynthesis differently among plant tissues, leading to distinct cardenolide expression in roots and shoots.

Furthermore, in contrast to the complex interactions that we observed among milkweed species, AMF inoculum availability, and herbivore species on foliar cardenolide expression, herbivores had simple and consistent effects on root cardenolides. Aphid feeding decreased root cardenolide concentrations, while caterpillar feeding increased them. Our findings support a growing body of work demonstrating pervasive, but differential, effects of aboveground herbivores on foliar and root defenses (Erb et al. 2008, Rasmann et al. 2009a, Huang et al. 2014, Mundim et al. 2017), but indicate that AMF further mediate these differential effects above and

belowground. Plant hormones play an integral role in the differential induction of defenses by herbivores in above and belowground plant tissues (Soler et al. 2013b, Papadopoulou and van Dam 2017), and AMF interact strongly with these same hormones (Gutjahr 2014, Bucher et al. 2014, Pozo et al. 2015). Therefore, the different effects of AMF availability on herbivore induction in leaves and roots may result from particular interactions between AMF and herbivores via their impacts on plant hormones above and belowground.

Of the limited studies that have considered the impact of aboveground herbivores on root defenses (Erb et al. 2008, 2015, Rasmann et al. 2009a, Huang et al. 2014, Vannette and Hunter 2014), most have considered only chewing herbivores (but see Huang et al. 2014, Vannette and Hunter 2014). Here, we demonstrate that a phloem-feeding herbivore can have stronger effects on root defenses than a chewing herbivore, with the relative strength of the effects maintained across plant species and levels of AMF inoculum availability. However, previous studies have found no effect of aphid feeding on concentrations of root defensive metabolites (Vannette and Hunter 2014, Huang et al. 2014), and therefore stronger effects of caterpillar herbivory than aphid herbivory (Huang et al. 2014). To better understand herbivore-induced plant resistance from a whole-plant perspective, more studies are needed that incorporate microbial root mutualists and assess the influence of herbivores with different feeding modes on both leaf and root defenses.

Herbivore effects on AMF colonization

In addition to the influence of AMF on herbivore-induction of plant defenses, we observed rapid and powerful effects of herbivore feeding on levels of AMF colonization. Here, aphid feeding generally decreased levels of AMF colonization, as has been found previously (Babikova et al. 2014), perhaps by inducing carbon-limitation in plants (Gehring and Whitham 1994, Gehring and Bennett 2009, Barto and Rillig 2010). However, aphid feeding increased AMF colonization of *A. incarnata* roots. Caterpillar feeding also had plant species-specific effects on AMF colonization, decreasing AMF colonization in *A. syriaca*, but increasing AMF colonization in *A. latifolia*. The plant species-specific effects of aphid and caterpillar feeding on AMF colonization may result from the varying ability of plant species to control carbon

allocation to AMF (Grman 2012), despite potentially being carbon-limited due to herbivore feeding.

Alternatively, herbivores may affect AMF colonization of roots by altering plant hormones that regulate AMF colonization (Barto and Rillig 2010, Pozo et al. 2015). Shoot herbivory alters root-hormone profiles (Fragoso et al. 2014), and moderate wounding of leaves may promote AMF colonization of roots by enhancing JA biosynthesis (Landgraf et al. 2012, He et al. 2017). Considering that caterpillar and aphid feeding alter phytohormones in leaves of milkweed plants in an herbivore and plant species-specific manner (Agrawal et al. 2014, Ali and Agrawal 2014), the influence of aphid and caterpillar feeding on root hormones may drive the herbivore and plant species-specific patterns of AMF colonization that we observed.

Conclusion

We demonstrate that AMF availability in soils affects herbivore-induction of plant defenses differently above and belowground, and that herbivore feeding alters AMF colonization of plant roots substantially. Because the availability of AMF inoculum varies on small scales (Carvalho et al. 2003, Wolfe et al. 2007), plants within a single population may experience substantial variation in AMF availability. As AMF availability mediates herbivore-induction of plant defenses, variation in AMF availability in soils may have community-wide consequences by altering plant phenotypes; herbivore-induction of plant defenses affects the subsequent colonization, performance, and population dynamics of additional herbivores (e.g. Underwood and Rausher 2002, van Zandt and Agrawal 2004, Karban 2011). Furthermore, by altering AMF colonization of roots, herbivores may affect the overall abundance of AMF in soil (Powell et al. 2009). Ultimately, this could feed back to affect plant defenses and the performance of herbivores. In addition, because AMF play a key role in ecosystem processes, such as global C cycling (Bago et al. 2000), P cycling (Jansa et al. 2011), and soil aggregation (Brito et al. 2008), the influence of herbivores on AMF could have ecosystem-level consequences. Future studies should consider how natural AMF abundances alter plant defensive phenotypes, plant-herbivore interactions, and plant-soil feedbacks at individual, community, and ecosystem scales.

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Table 2.1. Effects of plant species, arbuscular mycorrhizal fungi (AMF) inoculation, herbivore feeding, and their interaction on plant traits, including the proportion of roots colonized by AMF, natural log-transformed foliar cardenolide concentration (mg/g), foliar cardenolide diversity, foliar cardenolide polarity, leaf toughness (specific leaf mass, SLM; mg/cm^2), natural log-transformed latex exudation (mg), natural log-transformed root cardenolide concentration (mg/g), root cardenolide diversity, root cardenolide polarity, carbon (C) concentration (%), natural log-transformed C/N ratio, nitrogen (N) concentration (%), phosphorous (P) concentration (%), aboveground biomass (g), belowground biomass (g), and relative allocation of biomass to roots and shoots (root/shoot biomass ratio). Numbers represent F-values and P-values from general linear mixed models. Samples sizes ranged from 12-20 plants per treatment for aboveground defensive traits and biomass, 8-10 for foliar nutritive traits, and 8-10 for belowground traits (see text for details). After ensuring that all plants in the zero AMF treatment remained AMF-free, they were excluded from subsequent analyses of AMF colonization of roots as a dependent variable. *A. incarnata* produced no foliar cardenolides in this study, and was therefore excluded from foliar cardenolide analyses.

	Plant species		**AMF**		**Herbivore**	
	F	*P*	*F*	*P*	*F*	*P*
Root colonization by AMF						
Proportion of roots colonized	$F_{3,210}$=7.41	<0.0001***	$F_{1,210}$=1.12	0.2912	$F_{2,210}$=0.58	0.5605
Foliar defenses						
Foliar cardenolide concentration	$F_{2,449}$=685.58	<0.0001***	$F_{2,449}$=9.45	<0.0001***	$F_{2,449}$=4.22	0.0152*
Foliar cardenolide diversity	$F_{2,436}$=1088.73	<0.0001***	$F_{2,436}$=13.13	<0.0001***	$F_{2,436}$=3.89	0.0212*
Foliar cardenolide polarity	$F_{2,436}$=43.62	<0.0001***	$F_{2,436}$=0.03	0.9689	$F_{2,436}$=0.68	0.5064
Leaf toughness (SLM)	$F_{3,608}$=265.26	<0.0001***	$F_{2,608}$=16.1	<0.0001***	$F_{2,608}$=7.97	0.0004***
Latex exudation	$F_{3,598}$=217.09	<0.0001***	$F_{2,598}$=1.02	0.3616	$F_{2,598}$=1.68	0.1877
Root defenses						
Root cardenolide concentration	$F_{3,304}$=436.49	<0.0001***	$F_{2,304}$=0.64	0.5303	$F_{2,304}$=4.86	0.0084**
Root cardenolide diversity	$F_{3,304}$=602.93	<0.0001***	$F_{2,304}$=3.61	0.0282*	$F_{2,304}$=1.21	0.2983
Root cardenolide polarity	$F_{3,304}$=1595.21	<0.0001***	$F_{2,304}$=0.28	0.7579	$F_{2,304}$=0.55	0.5751
Foliar nutritive quality						
Foliar C concentration	$F_{3,316}$=4.44	0.0045**	$F_{2,316}$=1.82	0.1638	$F_{2,316}$=10.46	<0.0001***
Foliar C/N ratio	$F_{3,316}$=38.98	<0.0001***	$F_{2,316}$=6.05	0.0026**	$F_{2,316}$=2.17	0.1162
Foliar N concentration	$F_{3,316}$=30.09	<0.0001***	$F_{2,316}$=6.61	0.0015**	$F_{2,316}$=3.78	0.0239*
Foliar P concentration	$F_{3,315}$=27.26	<0.0001***	$F_{2,315}$=0.35	0.7061	$F_{2,315}$=6.48	0.0017**
Plant biomass						
Aboveground biomass	$F_{3,596}$=142.69	<0.0001***	$F_{2,596}$=6.55	0.0015**	$F_{2,596}$=0.83	0.4354
Belowground biomass	$F_{3,315}$=82.01	<0.0001***	$F_{2,315}$=15.9	<0.0001***	$F_{2,315}$=2.23	0.1097
Root/shoot biomass ratio	$F_{3,307}$=125.52	<0.0001***	$F_{2,307}$=1.3	0.2739	$F_{2,307}$=6.93	0.0011**

*** $P<0.001$; ** $P<0.01$, * $P<0.05$

Table 2.1, continued.

Plant species * AMF		**Plant species * Herbivore**		**AMF * Herbivore**		**Plant species * AMF * Herbivore**	
F	*P*	*F*	*P*	*F*	*P*	*F*	*P*
$F_{3,210}$=0.87	0.457	$F_{6,210}$=2.54	0.0216**	$F_{2,210}$=0.63	0.5325	$F_{6,210}$=0.49	0.814
$F_{4,449}$=1.35	0.2515	$F_{4,449}$=8.07	<0.0001***	$F_{4,449}$=0.51	0.7248	$F_{8,449}$=2.22	0.0251*
$F_{4,436}$=3.1	0.0156*	$F_{4,436}$=4.84	0.0008***	$F_{4,436}$=3.84	0.0045**	$F_{8,436}$=1.89	0.0595
$F_{4,436}$=1.88	0.1122	$F_{4,436}$=0.69	0.6011	$F_{4,436}$=0.57	0.6879	$F_{8,436}$=0.78	0.6224
$F_{6,608}$=4.97	<0.0001***	$F_{6,608}$=4.71	0.0001***	$F_{4,608}$=8.49	<0.0001***	$F_{12,608}$=1.91	0.0303*
$F_{6,598}$=2	0.0633	$F_{6,598}$=3.14	0.0049**	$F_{4,598}$=1.47	0.2108	$F_{12,598}$=1.06	0.3956
$F_{6,304}$=1.17	0.3224	$F_{6,304}$=0.31	0.9304	$F_{4,304}$=0.76	0.5532	$F_{12,304}$=0.83	0.6217
$F_{6,304}$=4.54	0.0002***	$F_{6,304}$=0.5	0.8058	$F_{4,304}$=1.5	0.2022	$F_{12,304}$=0.9	0.5426
$F_{6,304}$=1.79	0.1	$F_{6,304}$=1.13	0.3448	$F_{4,304}$=2.79	0.0265*	$F_{12,304}$=1.74	0.057
$F_{6,316}$=1.26	0.2762	$F_{6,316}$=1.07	0.3794	$F_{4,316}$=1.2	0.3097	$F_{12,316}$=1.16	0.3106
$F_{6,316}$=1.3	0.2547	$F_{6,316}$=0.33	0.9214	$F_{4,316}$=1.58	0.1805	$F_{12,316}$=1.27	0.2355
$F_{6,316}$=1.26	0.2767	$F_{6,316}$=0.32	0.9242	$F_{4,316}$=1.34	0.2551	$F_{12,316}$=0.96	0.4887
$F_{6,315}$=6.76	<0.0001***	$F_{6,315}$=0.8	0.5676	$F_{4,315}$=1.13	0.3439	$F_{12,315}$=0.97	0.4814
$F_{6,596}$=5.41	<0.0001***	$F_{6,596}$=0.94	0.4643	$F_{4,596}$=0.22	0.9299	$F_{12,596}$=0.68	0.7714
$F_{6,315}$=5.14	<0.0001***	$F_{6,315}$=0.71	0.6429	$F_{4,315}$=0.96	0.4304	$F_{12,315}$=0.77	0.6772
$F_{6,307}$=1.61	0.144	$F_{6,307}$=2.09	0.0539	$F_{4,307}$=0.81	0.5188	$F_{12,307}$=0.77	0.678

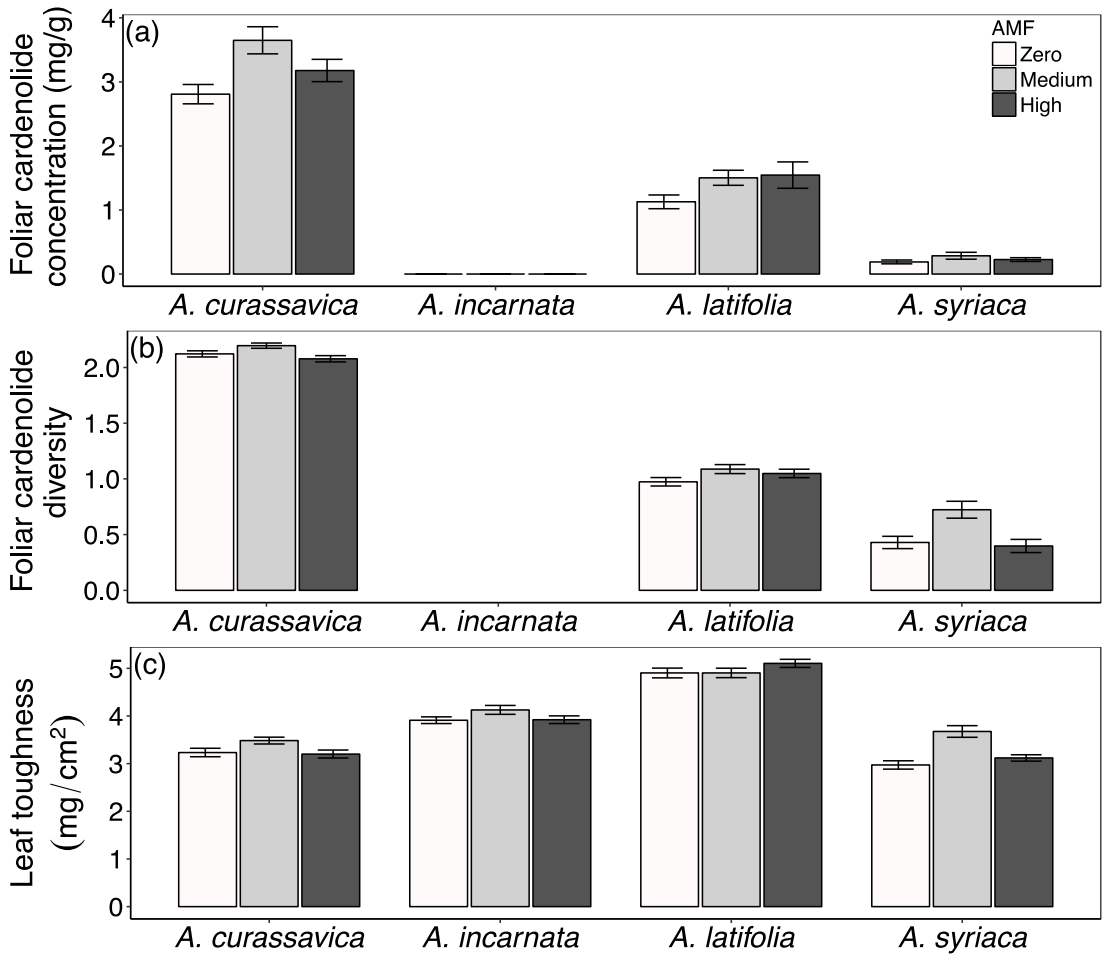

Figure 2.1. Effects of AMF inoculum availability (zero, medium, high) on foliar defenses, including a) foliar cardenolide concentrations, b) foliar cardenolide diversity, and c) leaf toughness (specific leaf mass) of four milkweed species. Sample sizes range from 53-54 plants for foliar cardenolide concentration, 48-54 plants for foliar cardenolide diversity, and 52-55 plants.

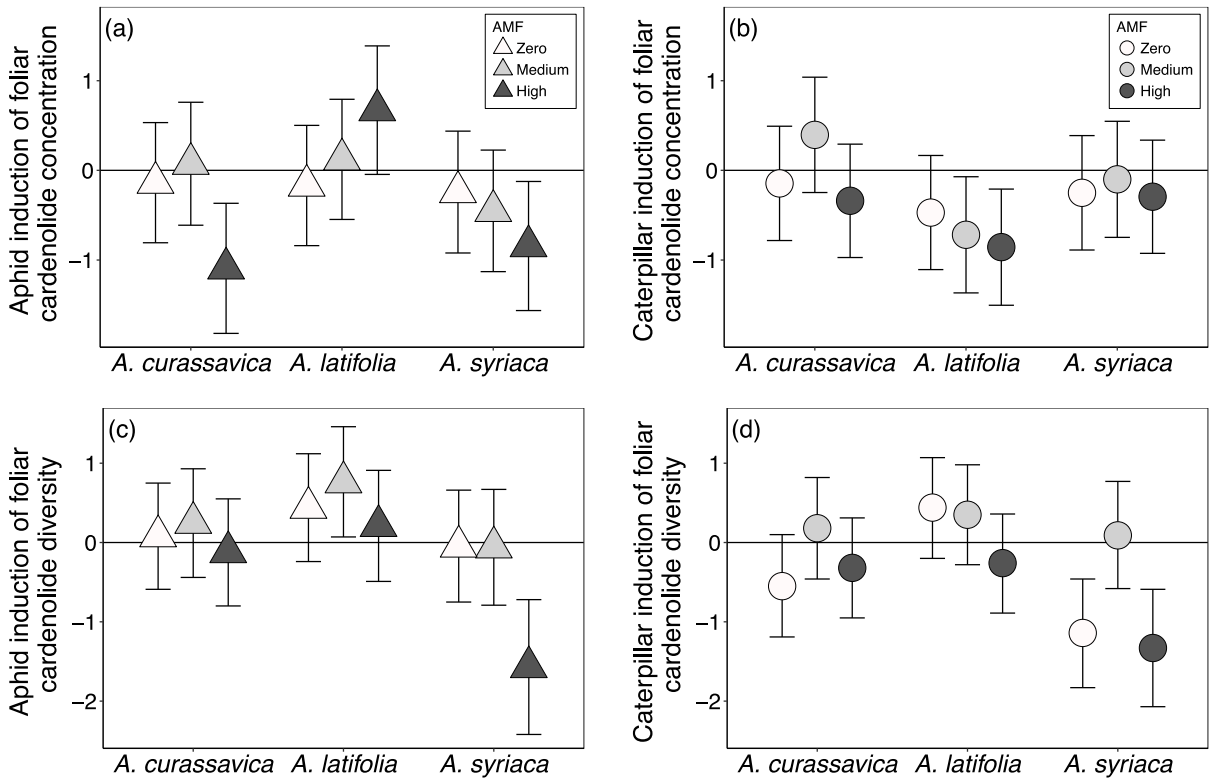

Figure 2.2. Influence of AMF inoculum availability (zero, medium, high) on the effect size of herbivore induction of foliar chemical defenses, including a) aphid and b) caterpillar induction of foliar cardenolide concentrations, and c) aphid and d) caterpillar induction of foliar cardenolide diversity. Effect size is calculated as Hedge's D, comparing mean and pooled standard deviation of herbivore damaged plants to control plants. Sample sizes range from 13-20 plants per plant species x AMF x herbivore treatment. Points display the effect size ± 95% confidence intervals. Points with confidence intervals above zero indicate that herbivore feeding increased foliar chemical defenses, whereas points with confidence intervals below zero indicate herbivore feeding suppressed defenses.

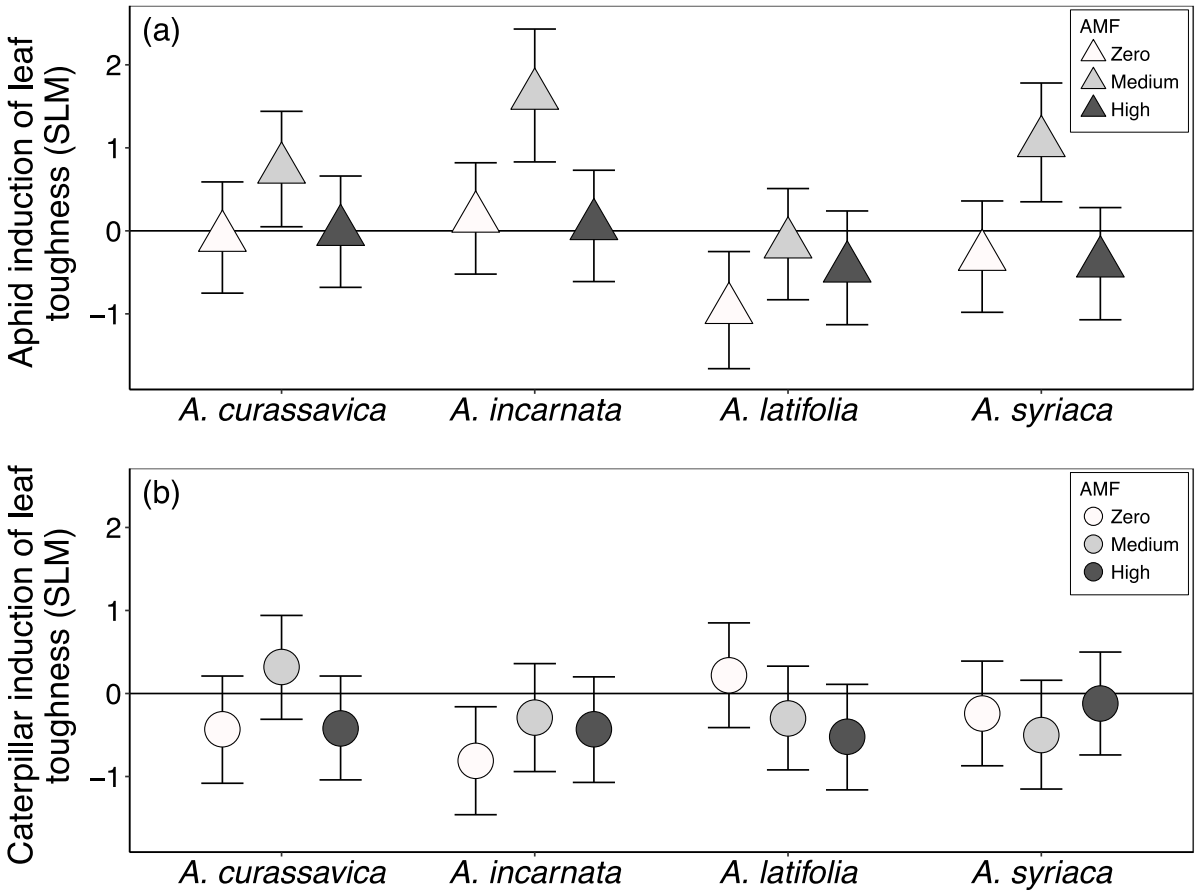

Figure 2.3. Influence of AMF inoculum availability (zero, medium, high) on the effect size of a) aphid and b) caterpillar induction of leaf toughness (SLM). Effect size is calculated as Hedge's D, comparing mean and pooled standard deviation of herbivore-damaged plants to control plants. Sample sizes range from 15-20 plants per plant species x AMF x herbivore treatment. Points display the effect size ± 95% confidence intervals. Points with confidence intervals above zero indicate that herbivore feeding increased leaf toughness, whereas points with confidence intervals below zero indicate herbivore feeding suppressed leaf toughness.

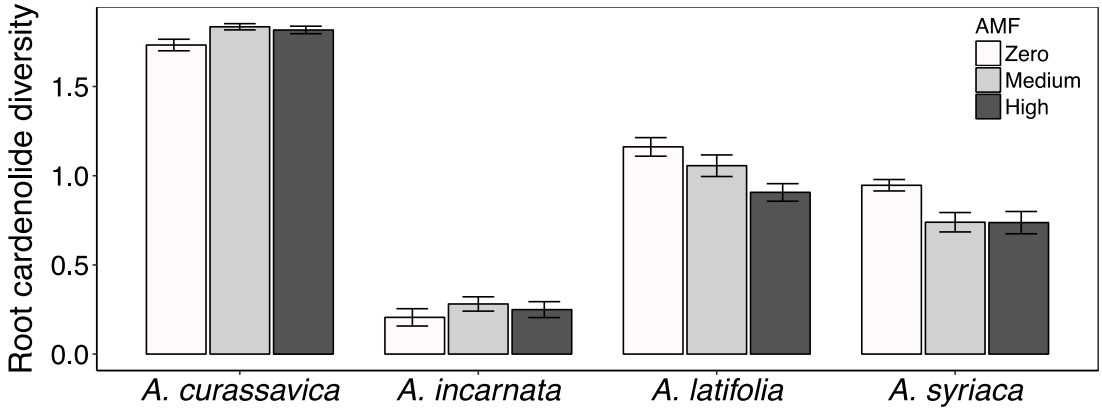

Figure 2.4. Effects of AMF inoculum availability (zero, medium, high) on root cardenolide diversity of four milkweed species. Samples sizes range from 28-30 plants per plant species x AMF treatment. Bars display the mean ± 1SE.

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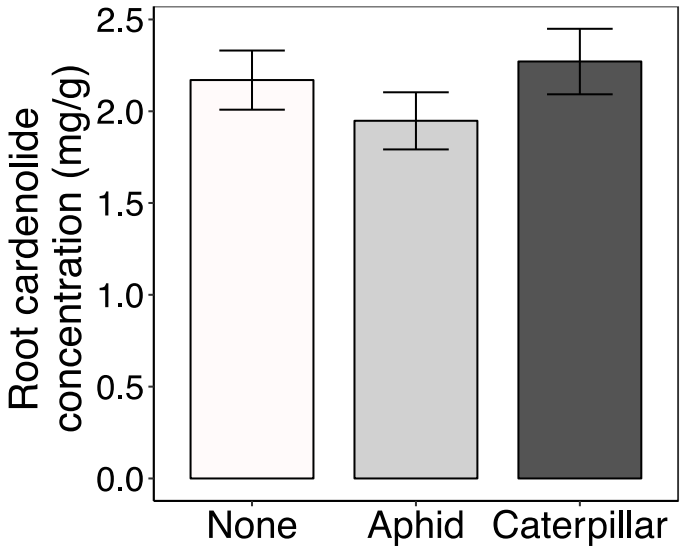

Figure 2.5. Effect of herbivore damage on root cardenolide concentrations of four milkweed species. Sample sizes range from 110-117 plants per herbivore treatment. Bars display the mean ± 1SE

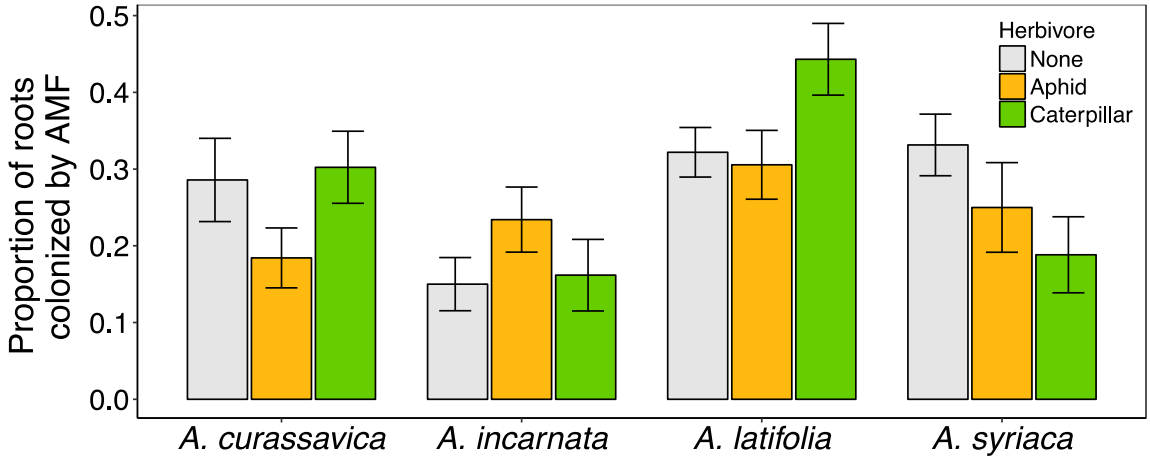

Figure 2.6. Effect of herbivore feeding on the proportion of roots colonized by AMF in four milkweed species. Plants that were not inoculated with live AMF (zero AMF treatment) were never colonized by AMF, and are not included in this analysis. Sample sizes range from 18-20 plants per plant species x herbivore treatment. Bars display the mean ± 1SE.

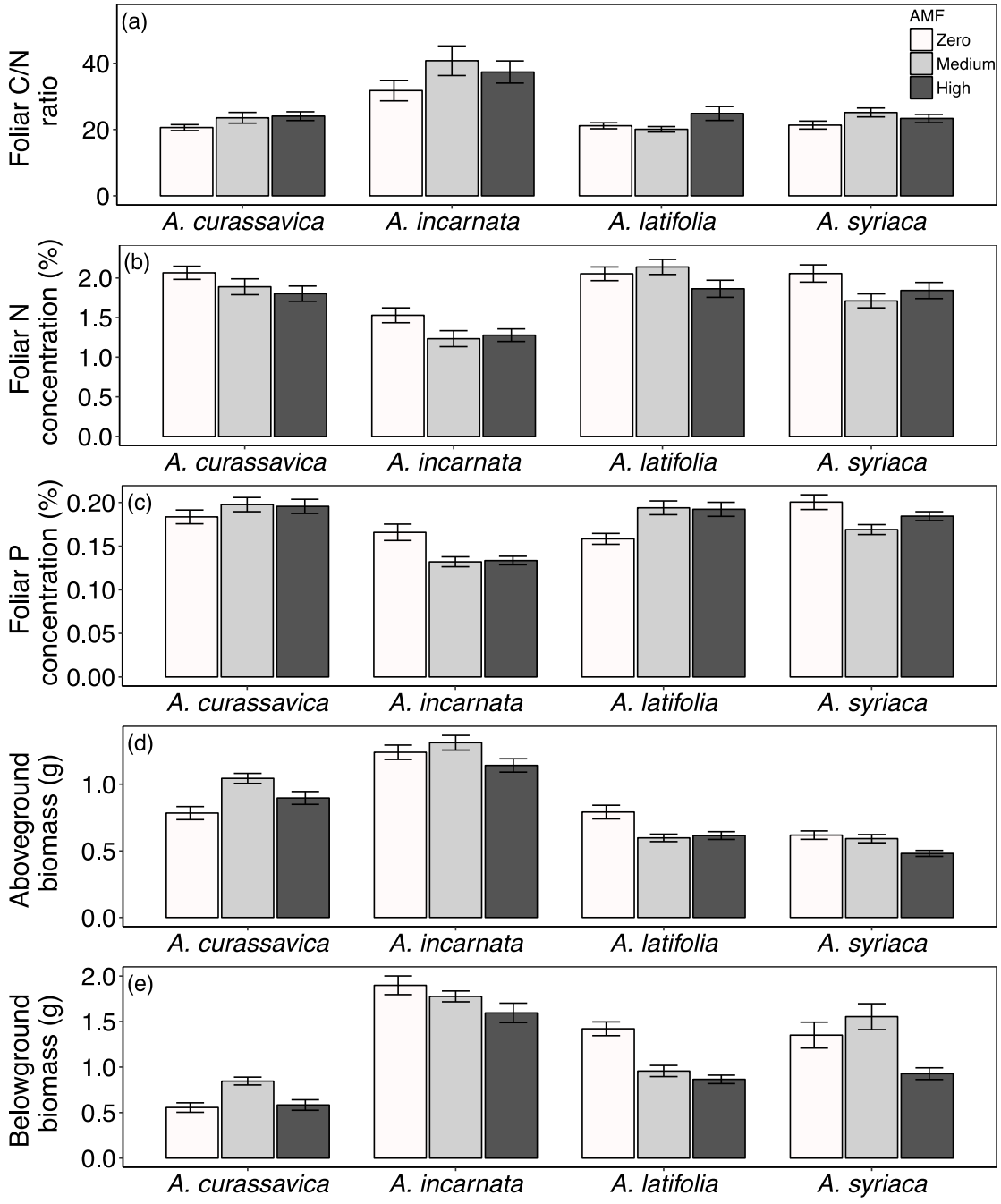

Figure 2.7. Effects of AMF inoculum availability (zero, medium, high) on a) foliar C/N ratio, b) foliar N concentration, c) foliar P concentration, d) aboveground biomass, and e) belowground biomass of four milkweed species. Sample sizes range from 28-30 plants per plant species x AMF treatment for foliar C, N, and P concentrations, 50-55 plants for aboveground biomass, and 29-30 plants for belowground biomass. Bars display the mean ± 1SE.

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Chapter III

Mycorrhizae alter toxin sequestration and performance of two specialist herbivores

Abstract

Multitrophic species interactions are shaped by both top-down and bottom-up factors. Belowground symbionts of plants, such as arbuscular mycorrhizal fungi (AMF), can alter the strength of these forces by altering plant phenotype. For example, AMF-mediated changes in foliar toxin and nutrient concentrations may influence herbivore growth and fecundity. In addition, many specialist herbivores sequester toxins from their host plants to resist natural enemies, and the extent of sequestration varies with host plant secondary chemistry. Therefore, by altering plant phenotype, AMF may affect both herbivore performance and their resistance to natural enemies. We examined how inoculation of plants with AMF influences toxin sequestration and performance of two specialist herbivores feeding upon four milkweed species (*Asclepias incarnata, A. curassavica, A. latifolia, A. syriaca*). We raised aphids (*Aphis nerii*) and caterpillars (*Danaus plexippus*) on plants for six days in a fully factorial manipulation of milkweed species and level of AMF inoculation (zero, medium, and high). We then assessed aphid and caterpillar sequestration of toxins (cardenolides) and performance, and measured defensive and nutritive traits of control plants.

Aphids and caterpillars sequestered higher concentrations of cardenolides from plants inoculated with AMF across all milkweed species. Aphid per capita growth rates and aphid body mass varied nonlinearly with increasing AMF inoculum availability; across all milkweed species, aphids had the lowest performance under medium levels of AMF availability and highest performance under high AMF availability. In contrast, caterpillar survival varied strongly with AMF availability in a plant species-specific manner, and caterpillar growth was unaffected by AMF. Inoculation with AMF increased foliar cardenolide concentrations consistently among milkweed species, but altered aboveground biomasses and foliar phosphorous concentrations in a plant species-specific fashion. Increased herbivore sequestration of cardenolides followed AMF-mediated increases in foliar cardenolide concentrations. Aphid performance declined with increasing foliar cardenolide concentrations, while caterpillar survival increased with

aboveground biomass. Our findings suggest that by altering plant phenotype, the availability of AMF in soil has the potential to influence both top-down (via sequestration) and bottom-up (via plant defense and nutrition) forces that operate on herbivores.

Introduction

Multitrophic species interactions are governed by a mixture of top-down forces, such as predators and parasites, and bottom-up forces, such as resource availability (Hunter and Price 1992, Schmitz et al. 2000). In terrestrial ecosystems, both top-down and bottom-up forces travel with ease across the traditional soil "boundary", with plants connecting the interactions that occur between above and belowground organisms (van der Putten et al. 2001, van Dam and Heil 2011, Hunter 2016). As a result, soil organisms that are associated with plant roots have the potential to affect herbivore populations above ground both by affecting plant quality for herbivores from the bottom-up (Hartley and Gange 2009, Koricheva et al. 2009, Jung et al. 2012) and the resistance of herbivores to their natural enemies from the top-down (Gange et al. 2003, Rasmann et al. 2017, Tao et al. 2017).

Arbuscular mycorrhizal fungi (AMF) engage in one of the most ubiquitous root-microbe symbioses in terrestrial ecosystems (Smith and Read 2008), associating with over eighty percent of plant species globally (Wang and Qiu 2006, Smith and Read 2008, Soudzilovskaia et al. 2015). AMF provide nutrients to plants, such as phosphorous, in exchange for plant sugars (Smith and Read 2008). In establishing and maintaining the symbiosis, AMF also interact with plant defensive signaling pathways, including the jasmonic acid and salicylic acid pathways (Jung et al. 2012, Cameron et al. 2013, Bucher et al. 2014, Gutjahr 2014). As a result, AMF alter plant nutritive quality and a diversity of plant primary and secondary metabolites (Bennett et al. 2009, Vannette et al. 2013, Roger et al. 2013, Schweiger et al. 2014, Schweiger and Müller 2015), affecting plant quality for insect herbivores substantially (Hartley and Gange 2009, Koricheva et al. 2009).

The response of insect herbivores to AMF colonization of their host plants varies widely, from positive to neutral or negative (Koricheva et al. 2009). Much of this variation is explained by the degree of specialization and feeding mode of the herbivore (Hartley and Gange 2009,

Koricheva et al. 2009). For instance, both generalist and specialist phloem-feeding insects, such as aphids, generally benefit from AMF colonization of their host plants. Specialist chewing herbivores, such as caterpillars, also benefit, but generalist chewing herbivores are negatively affected by AMF colonization of their host plants (Hartley and Gange 2009, Koricheva et al. 2009). Phloem-feeding insects may avoid AMF-mediated increases in plant defenses because phloem lacks or contains far lower concentrations of plant secondary metabolites than leaves (Züst and Agrawal 2016a). In addition, phloem-feeding insects may benefit from AMF-mediated increases in the size of plant vascular bundles (Krishna et al. 1981, Simon et al. 2017). Generalist chewers may be more susceptible to AMF-mediated increases in plant defenses (Schoonhoven et al. 2005), while specialist chewers may benefit from increased nutritive quality of host plants colonized by AMF (Koricheva et al. 2009).

Even within these trends, there is large variation in herbivore responses to AMF, and we lack an understanding of what is driving this variation. For instance, aphids generally benefit from AMF colonization of their host plants; aphids are more attracted to plants colonized by AMF and have greater body masses, growth rates, and fecundity on host plants colonized by AMF (Gange and West 1994, Gange et al. 1999, Gange et al. 2002, Koricheva et al. 2009, Babikova et al. 2014a, Babikova et al. 2014b, Simon et al. 2017). However, aphids have also been found to not respond to AMF colonization of their host plants (Pacovsky et al. 1985, Wurst et al. 2004, Colella et al. 2014, Grabmaier et al. 2014, Williams et al. 2014, Bennett et al. 2016) or to have reduced population growth on plants colonized by AMF (Gehring and Whitham 2002, Hempel et al. 2009, Abdelkarim et al. 2011). Similarly, while some specialist chewers benefit from AMF colonization of their host plants (Borowicz 1997, Goverde et al. 2000, Vannette and Hunter 2013), others are unaffected (Laird and Addicott 2008, Cosme et al. 2011). Some of this variation may be explained by the stage of the association between the plant and AMF; aphids, for example, tend to benefit only after at least a month of AMF establishment (Tomczak and Müller 2017). This variation in herbivore responses to AMF may also be a consequence of plant species-specific responses of plant traits to the presence of AMF (e.g. Grman 2012, Barber et al. 2013, Anacker et al. 2014, Tao et al. 2016a) and the density or identity of AMF inoculum available to the plant (Barber et al. 2013, Garrido et al. 2010, Vannette and Hunter 2011, Vannette and Hunter 2013).

In addition to being shaped by host plant quality, herbivore populations are also affected by their natural enemies. Root-associated microbes, such as AMF, affect herbivore-natural enemy interactions indirectly by altering plant phenotype (Rasmann et al. 2017, Tao et al. 2017). For instance, AMF increase the attractiveness of plants to natural enemies by changing the volatile emissions of their host plants (Guerrieri et al. 2004, Fontana et al. 2009, Hoffmann et al. 2011, Schausberger et al. 2012, Babikova et al. 2013). AMF also influence the searching efficiency of natural enemies, likely by changing plant size (Gange et al. 2003), and can improve natural enemy performance (Hempel et al. 2009, Bennett et al. 2016). AMF mediation of herbivore-natural enemy interactions can ultimately benefit host plants. For instance, AMF colonization increases herbivorous mite densities on *Phaseolus vulgaris* plants, yet improves plant productivity by enhancing the population growth of predatory mites and plant tolerance sufficiently to compensate for the increase in herbivores (Hoffmann et al. 2011).

Many specialist herbivores are able to resist their natural enemies by sequestering secondary metabolites from their host plants (Nishida 2002, Opitz and Müller 2009, Ode 2013, Erb and Robert 2016, Petschenka and Agrawal 2016). The concentration and composition of secondary metabolites that herbivores sequester are tied closely with host plant secondary chemical profiles (Malcolm 1990, Malcolm 1994, Agrawal et al. 2015, Petschenka and Agrawal 2015), and are affected by environmental factors, such as soil nutrient availability (Jamieson and Bowers 2012, Tao and Hunter 2015). Herbivores that sequester higher concentrations of secondary metabolites from their host plants are more toxic and deterrent to their natural enemies (Brower et al. 1968, Reichstein et al. 1968, Brower and Moffitt 1974, Malcolm 1992, Dyer and Bowers 1996, Camara 1997). Therefore, by increasing plant chemical defenses, AMF may increase toxin sequestration by herbivores, thereby improving herbivore resistance to their natural enemies. Despite widespread recognition of sequestration as an integral component of host plant specialization and an important factor shaping ecological networks (Duffey 1980, Lampert et al. 2014, Züst and Agrawal 2016b, Petschenka and Agrawal 2016), no study to date has considered how microbial root mutualists of plants, including AMF, affect herbivore sequestration of plant toxins.

Here, we evaluate how AMF affect toxin sequestration and performance of specialist herbivores of milkweed (*Asclepias*) species. Milkweed species provide an ideal system in which to address these questions because milkweed species produce a suite of resistance traits and are fed upon by specialized herbivores that can tolerate and sequester milkweed defenses. Milkweed tissues, including leaves and phloem, contain cardenolides, bitter tasting steroids that disrupt the functioning of sodium-potassium channels in animal cells by inhibiting an essential cation transporter, Na^+/K^+-ATPase (Agrawal et al. 2012, Pringle et al. 2014, Züst and Agrawal 2016b). In response to leaf damage, milkweeds exude latex, a sticky isoprene polymer that gums up the mouths of chewing herbivores (Zalucki et al. 2001a, Agrawal and Konno 2009). In addition, milkweed species vary in leaf toughness (Agrawal and Fishbein 2006), which is tightly correlated with specific leaf mass (SLM) (Frost and Hunter 2008).

We used two specialist herbivores of milkweed that vary in their feeding mode: oleander aphids (*Aphis nerii*; phloem-feeding) and monarch caterpillars (*Danaus plexippus*; leaf-chewing). Oleander aphids tolerate cardenolides through regulation of a narrow set of genes involved in canonical detoxification processes (Birnbaum et al. 2017). Monarch caterpillars, in contrast, have NA^+/K^+- ATPases that are insensitive to cardenolides (Dobler et al. 2012, Petschenka and Agrawal 2015). Despite being able to tolerate cardenolides, both oleander aphids and monarch caterpillars exhibit reduced performance on host plants with high concentrations of cardenolides (Zalucki et al. 2001a, Agrawal 2004, Agrawal 2005, Rasmann et al. 2009, de Roode et al. 2011, Colvin et al. 2013, Tao et al. 2016b, Birnbaum et al. 2017). Furthermore, both oleander aphids and monarch caterpillars sequester cardenolides (Rothschild et al. 1970, Malcolm and Brower 1989, Malcolm 1990, Züst and Agrawal 2016b), providing an effective defense against aphid predators (Pasteels 1978, Malcolm 1989, Malcolm 1992, Pappas et al. 2007, Mooney et al. 2008) and monarch predators and parasites (Brower et al. 1968; Reichstein et al. 1968, Brower and Moffitt 1974, Sternberg et al. 2012). Oleander aphids appear to sequester cardenolides passively through diffusion of nonpolar (lipophilic) cardenolides (Malcolm 1990, Züst and Agrawal 2016b). In contrast, monarch caterpillars sequester polar cardenolides selectively (Malcolm and Brower 1989, Tao and Hunter 2015, Petschenka and Agrawal 2015, Erb and Robert 2016), likely through active translocation by transport proteins through gut membranes (Frick and Wink 1995). Nonetheless, cardenolide sequestration by both oleander

aphids and monarch caterpillars is closely correlated with their host plant cardenolides (Malcolm 1990, Malcolm 1994, Agrawal et al. 2015, Petschenka and Agrawal 2015). Thus, AMF-mediated changes in plant cardenolide expression may influence aphid and caterpillar sequestration.

We performed a full-factorial experiment, manipulating oleander aphids and monarch caterpillars on four closely related milkweed species provided with different amounts of AMF inoculum. We expected herbivores to sequester higher concentrations of cardenolides on AMF-colonized plants due to AMF-mediated increases in the cardenolide concentrations of their host plants. Furthermore, we expected that AMF colonization would improve the performance of aphids and caterpillars by increasing plant nutritive quality and biomass, outweighing the negative effects of increased cardenolide concentrations on the herbivores. Because the outcomes of many AMF-plant associations are specific to the AMF and plant species (e.g. Grman 2012, Barber et al. 2013, Anacker et al. 2014, Tao et al. 2016a), we expected the magnitude of the effects of AMF on herbivore sequestration and performance to vary among plant species and with the level of AMF inoculum available to the plant.

Materials and methods

Plants and insects

We used four North American milkweed species (*Asclepias curassavica, A. latifolia, A. syriaca*, and *A. incarnata)* that show constitutive and AMF-mediated variation in milkweed defenses and nutritive quality (Vannette et al. 2013, Tao et al. 2016a). *Asclepias incarnata* and *A. syriaca* seeds were collected from naturally occurring populations in Livingston County, MI, and *A. latifolia* and *A. curassavica* seeds were purchased from commercial sources (Alplains and Butterfly Encounters Inc., respectively). We obtained fungal inoculum from Mycorrhizal Applications (Grants Pass, OR, USA), which was comprised of equal proportions of four AMF species including *Rhizophagus intraradices*, *Funneliformis mosseae*, *Glomus aggregatum*, and *Claroideoglomus etunicatum* (33 spores of each AMF species per gram of inoculum, www.plant-success.com). However, cloning and sequencing of the inoculum with AMF-specific primers (Krüger et al. 2009) revealed the mix to consist only of *F. mosseae* (details in Appendix B). Milkweeds grow in habitats that host a diversity of AMF taxa (Öpik et al. 2006), and can form associations with these cosmopolitan AMF species in natural and experimental populations

(Landis et al. 2004, Vannette and Hunter 2011, Vannette et al. 2013, Tao et al. 2015, Tao et al. 2016a). However, as with most systems, the frequency of these relationships is not known.

To assess how the availability of AMF inoculum influences the performance of herbivores, we used two specialist herbivores: oleander aphids (*Aphis nerii;* phloem-feeding) and monarch caterpillars (*Danaus plexippus;* leaf-chewing). All oleander aphids used in the experiment were clones derived from a single aphid collected in March 2014 from the Emory University greenhouses (Atlanta, GA) and reared indoors on *A. tuberosa*, which does not produce cardenolides, for 1 month prior the experiment. Monarch larvae were the second generation of outcrossed progeny of butterflies obtained from Shady Oak Farms (www.butterfliesetc.com), Mr. Butterfly (www.mrbutterflies.com), and Butterfly Release Company (www.butterflyreleasecompany.com). Monarch larvae were raised on a combination of *A. syriaca*, *A. incarnata*, and *A. curassavica* in a growth room with photoperiod of 16:8 L:D and adults were reared on a 10% honey solution.

Experimental protocols

After six weeks of cold, moist stratification at 4 °C, we surface-sterilized seeds in 5% bleach and germinated them at room temperature (*A. curassavica* did not require stratification) in March 2014. We planted individual seedlings in conical deepots (D40H, Steuwe and Sons Inc., Corvalis, OR, USA) filled with 600 ml of a 3:1 mix of autoclaved soil (Metro-Mix 380; MetroMix Sun Gro Horticulture Canada CM Ltd., Vancouver, BC, Canada) and sand containing AMF inoculum. We manipulated the amount of live and autoclaved (dead) AMF inoculum available to experimental plants to generate zero, medium, and high levels of root colonization, which is possible because the amount of AMF inoculum available to milkweed plants affects the levels of AMF colonization of roots (Vannette and Hunter 2011, Tao et al. 2015, Tao et al. 2016a). Specifically, we homogenized 4.20 g autoclaved AMF inoculum (zero treatment), 1.20 g live and 3.00 g autoclaved inoculum (medium treatment), or 4.20 g live inoculum (high treatment) in 200 ml of autoclaved soil, which was placed between 400 ml of autoclaved soil and sand to prevent the transfer of mycorrhizal spores or hyphae among treatments. To return the natural bacterial community of the potting soil to the autoclaved soil of each pot, we added 20 ml of bacterial solution made by suspending 100 ml potting soil in 1 L deionized water and filtering

the suspension through an ultra-fine soil sieve (38 µm) to remove AMF hyphae and spores. Plants were grown at the Matthaei Botanical Gardens greenhouses (Ann Arbor, MI) with a photoperiod of 16:8 L:D for three months. Plants were watered *ad libitum* and fertilized biweekly with 90 ml of a low concentration (94 ppm) of 15-0-15 (N-P-K) dark weather fertilizer (JR Peters Inc., Allentown, PA). All experimental plants were exposed to colonization and damage by greenhouse thrips and sprayed monthly with a mixture of Enstar, Lucid, and MPede to minimize damage. No pesticides were sprayed for three weeks prior to the addition of herbivores; thrips were killed weekly by hand during this period.

In a fully factorial design, we placed oleander aphids, monarch caterpillars, or no herbivores on plants of each plant species x AMF treatment and allowed herbivores to feed for six days in June 2014. The six days of feeding represent approximately one generation for oleander aphids (Zehnder and Hunter 2009) and 50% of the average larval period of monarchs under our rearing conditions (Vannette and Hunter 2013). Effects of plant quality on monarch growth are most important during early instars (Zalucki et al. 2001b). All plants were covered with white, nylon mesh bags (5-gallon paint strainer bags) to prevent insect movement among experimental plants. Five reproductive, apterous oleander aphids were placed at the apex of 15 replicates of each plant species x AMF treatment and allowed to reproduce naturally for six days (n=180). Dead or missing reproductive aphids were replaced on the second day. One newly hatched monarch caterpillar was placed on each of 20 replicates of each plant species x AMF treatment and allowed to feed for six days (n=240). Missing or dead caterpillars were replaced on the second day. Twenty plants of each plant species x AMF treatment experienced no herbivory but were covered with white, nylon mesh bags to control for effects of mesh on plant traits (n=240). We used these control plants to evaluate the effects of AMF on plant traits that may influence herbivore performance, and to determine the levels of AMF colonization of plant roots (n=240). We could not use the plants upon which the herbivores fed, because aphid and caterpillar feeding alters milkweed defenses, nutritive quality, and levels of AMF colonization (Meier and Hunter 2018). Therefore, the traits measured in herbivore-damaged plants would not be representative of the initial plant quality experienced by aphids and caterpillars. We conducted this experiment in four temporal blocks separated by one day, with each treatment equally represented in each temporal block.

Analysis of herbivore traits

After the six days, aphids were counted and collected, allowed to void their guts for 24 hours, frozen, lyophilized, and weighed. Caterpillars were also collected, allowed to void their guts for twenty-four hours, frozen, dried at 50 °C, and weighed. Simultaneously, control plants were harvested destructively to measure plant resistance and nutritive traits, biomass, and AMF colonization of roots. Aphid per capita growth rate per plant (r) was calculated by taking the natural log of the final aphid population size divided by the initial aphid population size (5 aphids) (Speight et al. 2008). Aphid individual mass was calculated by weighing each aphid population (i.e. all aphids present on one experimental plant) and dividing by the number of aphids in the population. Mean caterpillar growth rate per day was calculated by dividing the final, dry caterpillar mass by the six days for which it fed (Waldbauer 1968). Leaves damaged by caterpillars were removed, scanned, and the area consumed by caterpillars (consumed leaf area, CLA) was determined with Image J (Schneider et al. 2012, Roger et al. 2013). To calculate the efficiency of conversion of ingested biomass (ECI) for caterpillars, we first determined the mass of leaves consumed by caterpillars. To do so, we calculated a mass/area ratio per plant by weighing and photographing two to three dried leaves from leaf pairs neighboring those consumed by caterpillars, and measuring the leaf area using Image J. Using this mass/area ratio, we calculated the mass of leaves consumed by caterpillars from the consumed leaf area that we measured. We calculated ECI per caterpillar as the final dry mass of the caterpillar divided by the dry mass of food it consumed (Waldbauer 1968). Nine caterpillars that consumed flower buds in addition to leaves on *A. curassavica* plants were excluded from analyses of CLA and ECI. No other plant species produced flowers during the experiment.

After being dried and weighed, aphid populations and individual caterpillars were placed in 1 mL of methanol and stored at -10 °C until cardenolide analysis. We assessed the cardenolides that herbivores sequestered following well-established methods (Tao and Hunter 2015, Zehnder and Hunter 2007). Aphids and caterpillars were ground for 3 min in methanol, sonicated for 1 h, and then centrifuged for 6 min. The supernatant was evaporated under vacuum at 45°C until dry and resuspended in 150 µl methanol containing 0.15 mg ml^{-1} digitoxin as an internal standard. Samples were then separated by ultra-performance liquid chromatography

(UPLC; Waters Inc., Milford, MA, USA) using a Luna 2.5 μm C18(2) column (50 ~ 2 mm, Phenomenex Inc., Torrance, CA, USA). Each 2 μl injection was eluted at a constant flow of 0.7 ml per min with a gradient of acetonitrile and water for the 9 min run, maintaining first at 20% acetonitrile for 3 min, increasing to 45% acetonitrile for 5 min, and then maintaining at 20% acetonitrile for 1 min. Peaks were detected by a diode array detector at 218 nm, and absorbance spectra recorded from 200 to 400 nm. Symmetric peaks with maximum absorbance between 217 - 222 nm were quantified as cardenolides. Cardenolide concentrations were calculated using the digitoxin internal standard and total cardenolide concentrations were calculated as the sum of individual peaks. The masses of some aphid populations were too small to obtain enough dried material to detect cardenolides, and those samples were not included in our analyses of cardenolide sequestration (Table B3.1). In total, we analyzed 107 aphid populations (=replicate plants) with masses from 1.0 mg to 13.3 mg.

Analysis of plant traits

To measure foliar traits, we punched three fresh leaf disks from each leaf of the sixth leaf pair (six hole punches, 424 mm^2 total) of each plant, placed the disks in 1 mL of methanol, and stored them at -10 °C until cardenolide analysis. Foliar cardenolide concentrations were later assessed following the same procedure as for aphids and caterpillars (above). Latex that exuded from the hole punches was collected on pre-weighed cellulose disks, dried at 50 °C, and weighed. Six additional leaf disks were taken from the same leaves, stored in glassine envelopes, and dried at 50 °C. These leaf disks were weighed to estimate SLM and dry mass of foliar material used in cardenolide analyses. SLM was estimated by dividing the mass of dried leaf disks by the total disk area as a proxy for leaf toughness (Frost and Hunter 2008). Additional leaves from neighboring leaf pairs were removed and dried at 50 °C for subsequent carbon, nitrogen, and phosphorus analyses. Remaining plant material was dried at 50 °C in paper bags and weighed to measure aboveground biomass after correcting for foliar tissue removed for chemistry sampling.

Carbon (C) and nitrogen (N) concentrations of foliar tissues were measured with a TruMac elemental analyzer (Leco Corporation, St. Joseph, MI, 49085, USA). Phosphorous (P) concentrations of foliar samples were determined by dry combusting ground samples in a muffle

furnace at 550 °C overnight, followed by persulfate digestion at 121 °C for 60 min in an autoclave, and analysis by the molybdenum blue method on a PowerWave XS plate reader reading at 880 nm (Bio-Tek, Highland Park, Winooski, Vermont, 05404, USA). We calculated P concentrations of samples from a potassium phosphate standard curve and assessed quality control with NIST apple leaf standard analyzed with all samples. Only a subset of all experimental treatments were analyzed for nutritive traits, due to time and financial constraints (10 replicates of each plant species x AMF treatment, n=120).

After washing the roots in deionized water, we stored 150 mg of 1 cm pieces of fresh fine root tissue in 60% ethanol at 4 °C until we could quantify AMF colonization. We also took 400 mg of fresh fine root, dried it at 50 °C, and reweighed it to calculate wet weight/dry weight ratios from which to estimate the dry mass of the subsample taken to quantify AMF colonization. We dried all remaining root tissue at 50 °C and weighed its contribution to total root biomass. We analyzed a subset of roots of all experimental treatments (10 replicates of each AMF x plant species treatment, n=120) due to time constraints in harvesting.

To quantify AMF colonization, roots were cleared with 10% KOH for 10 min, acidified using 2% HCl, and stained in 0.05% trypan blue in 1:1:1 water:glycerol:lactic acid (Vannette and Hunter 2011). We mounted stained roots on slides and scored AMF colonization using the magnified gridline intersect method (McGonigle et al. 1990) with a Nikon compound microscope (Melville, NY, USA). A root intersection was considered colonized if hyphae, arbuscules, or vesicles were present. At least 100 root intersections were analyzed per plant.

Data Analyses

Some aphid populations did not sequester detectable concentrations of cardenolides on plants that contained cardenolides (Table B3.1), so we first determined whether the probability that aphids would sequester cardenolides was a function of plant species, AMF inoculum availability, or their interaction using a generalized linear mixed model with a binomial distribution and logit link function. Unlike aphids, all caterpillars sequestered cardenolides, except for those feeding on *A. incarnata*, so we did not evaluate the probability of caterpillar sequestration. For the aphid populations that did sequester cardenolides and all individual

caterpillars, we used general linear mixed models to evaluate the effects of AMF inoculum availability and milkweed species on herbivore sequestration. In all models, temporal block was a random effect while milkweed species, AMF inoculum availability, and their interaction were fixed effects. For monarchs, we also included the family from which the caterpillar originated as a random effect. Using these models, we evaluated the effects of AMF inoculum availability on three measures of cardenolides sequestered by herbivores; total cardenolide concentration (sum of all cardenolide peaks), cardenolide diversity (using Shannon's index), and cardenolide polarity (relative representation of lipophilic cardenolides), calculated by summing the relative peak areas multiplied by each peaks' retention time (Rasmann and Agrawal 2011, Sternberg et al. 2012). A greater diversity of cardenolides and more lipophilic cardenolides are considered more toxic than lower diversity or more polar mixes (Fordyce and Malcolm 2000, Zehnder and Hunter 2007, Sternberg et al. 2012). Because herbivores feeding upon *A. incarnata* rarely sequestered cardenolides (Table B3.1), they were excluded from all sequestration analyses.

For these and the following analyses, data were natural log- and log-transformed when necessary. In addition, we used Tukey's adjustment for multiple comparisons to identify significant differences among treatment means. We considered differences to be significant at $P < 0.05$, except when evaluating differences among AMF treatments within plant species. For these analyses, we considered differences to be significant at $P < 0.1$ due to the reduced sample size of these analyses. All statistical analyses were performed in SAS 9.4 (SAS Institute, Cary, NC, USA). Because several caterpillars died before the end of the experiment and several samples were lost during processing and chemical analyses, final sample sizes were smaller than initial (details in Table B3.2).

We also tested for differences in the composition (i.e. identity and relative abundance) of cardenolides sequestered by herbivores and present in leaves, among plant species, AMF treatments, and their interaction using permutational multivariate ANOVA (PERMANOVA; McCune et al. 2002). We used the adonis function in the vegan package (Oksanen et al. 2016) in R v 3.3.1 and calculated dissimilarities among samples using the Bray-Curtis metric for PERMANOVA. To evaluate how AMF influenced the composition of cardenolides in herbivore

and foliar tissue, we used non-metric multidimensional scaling (NMDS) through the vegan package.

We also used general linear mixed models to compare the effects of AMF inoculum availability and milkweed species on aphid and caterpillar performance. As before, temporal block was a random effect while AMF inoculum availability, milkweed species, and their interaction were fixed effects. For monarchs, we included the family from which the caterpillar originated as a random effect. Each herbivore performance measure (aphid per capita growth rate, aphid mass per individual, caterpillar growth rate, ECI, CLA) was a dependent variable. Not all caterpillars survived through the sixth day of feeding, so we assessed the probability of caterpillar survival among treatments using a generalized linear mixed model with a binomial distribution and logit link function.

We used general linear mixed models to evaluate the effects of AMF inoculum availability and milkweed species on plant traits. In all models, temporal block was a random effect while milkweed species, AMF inoculum availability, and their interaction were fixed effects. Each plant trait (i.e. foliar defensive traits, foliar nutritive traits, aboveground biomass, and levels of AMF colonization of roots) was a dependent variable. *A. incarnata* plants produced no foliar cardenolides in this study, and were therefore excluded from analyses of foliar cardenolides.

To gain some insight into the phenotypic traits of plants through which AMF influenced herbivores, we assessed the effects of measured plant traits on herbivore performance and sequestration using multiple regression. However, because herbivore and plant traits were measured from different groups of plants (above), we could only assess relationships among average values for each plant species x AMF treatment, yielding only 8-12 data points for these analyses. Therefore, we present these analyses in Appendix B.

Results

We summarize the effects of milkweed species, AMF inoculum availability, and their interaction on plant traits and herbivore traits (toxin sequestration and performance) in Tables 3.1 and 3.2, respectively. We describe key results in more detail below.

AMF colonization

The proportion of roots colonized by AMF arbuscules was tightly correlated with root colonization by all fungal structures (R^2=0.95, P<0.0001), so we report only the latter here. Inoculation with AMF led to successful root colonization, while control plants remained AMF-free ($F_{2,106}$=43.91, P<0.0001). Analysis of plants inoculated with live AMF (medium and high AMF treatments only) illustrated that AMF colonization was not a simple function of inoculum availability. Rather, levels of colonization varied substantially among plant species ($F_{3,70}$=4.00, P=0.011; Fig. B3.1), but were similar in medium and high AMF treatments ($F_{1,70}$=0.56, P=0.4586; Fig. B3.1). However, because herbivore performance varied substantially between medium and high AMF treatments (below), we conclude that the availability of inoculum had effects on plant phenotype beyond those observed by estimates of colonization alone. We have therefore continued to treat medium and high AMF treatments separately in all following analyses.

Herbivore sequestration of cardenolides

As expected (Malcolm 1990, Malcolm 1994, Agrawal et al. 2015, Petschenka and Agrawal 2015), the concentration, diversity, polarity, and composition of cardenolides sequestered by aphids and caterpillars varied strongly among plant species, following plant species-specific differences in cardenolide expression (Table 3.2, PERMANOVA aphid: Plant species $F_{2,50}$=22.2694, P<0.001; caterpillar: Plant species $F_{2,110}$=98.086, P<0.001). For instance, aphids and caterpillars sequestered the highest cardenolide concentration and diversity, and most lipophilic (non-polar) cardenolides, when feeding upon the high cardenolide-containing *A. curassavica* and the least when feeding upon the low cardenolide-containing *A. syriaca.*

Importantly, the amount of AMF inoculum available to the milkweed hosts of aphids and caterpillars influenced the concentration of cardenolides that aphid populations and caterpillars sequestered (aphid: AMF $F_{2,48}$=3.35, P=0.0434; Fig. 3.1a; caterpillar: AMF $F_{2,100}$=4.05,

P=0.0203; Fig. 3.1b). Across milkweed species, aphids sequestered, on average, 87% and 36% higher cardenolide concentrations when feeding upon plants under medium and high AMF availability, respectively, than when feeding upon plants without AMF (Fig. 3.1a). Similarly, caterpillars sequestered 38% and 25% higher cardenolide concentrations when they fed upon plants under medium and high AMF inoculum availability, respectively, than caterpillars that fed upon plants without AMF (Fig. 3.1b). The probability that aphid populations would sequester cardenolides did not vary among plant species or with AMF inoculum availability (Plant species $F_{2,93}$=2.56, P=0.0824; AMF $F_{2,93}$=0.65, P=0.5264).

The availability of AMF inoculum also shifted the community of cardenolides that aphids and caterpillars sequestered (PERMANOVA aphid: AMF $F_{2,50}$ = 2.2045, P=0.047; caterpillar: Plant species*AMF $F_{4,110}$=2.022, P=0.035). In addition, caterpillars feeding on plants under high AMF availability sequestered more diverse communities of cardenolides, by an average of 23%, than did caterpillars feeding upon plants under zero or medium AMF availability (AMF $F_{2,100}$=4.07, P=0.02; Fig. 3.1c). There were also minor, plant species-specific effects of AMF on the polarity of cardenolides that caterpillars sequestered (Plant species*AMF $F_{4,100}$=2.96, P=0.0234). Caterpillars sequestered 22% more lipophilic (non-polar) cardenolides when feeding upon *A. syriaca* plants under high AMF availability than on *A. syriaca* plants under zero or medium AMF availability. However, the polarity of cardenolides that caterpillars sequestered was unaffected by the amount of AMF available to *A. curassavica* and *A. latifolia*. AMF availability also did not influence the diversity or polarity of cardenolides that aphids sequestered (Table 3.2).

Herbivore performance

Aphid performance varied nonlinearly with increasing AMF availability; it was lowest on plants under medium AMF availability, but highest on plants under high AMF availability (Table 3.2, Fig. 3.2). Specifically, aphid per capita growth rates were 19% greater under high AMF availability than under medium AMF availability, with intermediate per capita growth rates on plants without AMF ($F_{2,166}$=13.09, P<0.0001; Fig. 3.2a). Similarly, individual aphids were 24% heavier on plants under high AMF availability than were aphids on plants under medium AMF availability ($F_{2,159}$=8.74, P=0.0003; Fig. 3.2b). As expected from previous work (Agrawal 2004),

aphid per capita growth rates and masses varied among milkweed species (r: $F_{3,166}$=9.10, P<0.0001; mass: $F_{3,159}$=28.62, P<0.0001).

The availability of AMF inoculum had striking effects on caterpillar survival, but those effects varied among milkweed species (Plant species*AMF χ^2=14.1, df=6, P=0.0286; Fig. 3.3). For example, caterpillars feeding on *A. incarnata* and *A. syriaca* were 13% and 44% more likely to survive on plants without AMF than on plants with AMF, respectively. In contrast, caterpillars feeding on *A. latifolia* were 38% more likely to survive on plants grown under medium AMF inoculum availability than on plants without AMF. Caterpillars feeding on *A. curasssavica* were affected minimally by AMF inoculum availability (Fig. 3.3). Caterpillar growth rates, efficiency of conversion of ingested biomass (ECI), and consumption of leaf area (CLA) varied widely among milkweed species, but were unaffected by the availability of AMF inoculum (Table 3.2).

Effects of AMF on plant traits

Consistent with the effects of AMF on cardenolide sequestration by herbivores (above), foliar cardenolide concentrations in milkweed plants under medium and high AMF availability were an average of 19% greater than were concentrations in AMF-free plants (AMF $F_{2,163}$=2.98, P=0.0538; Fig. 3.4). As expected (Rasmann and Agrawal 2011, Sternberg et al. 2012, Vannette et al. 2013), milkweed species varied in the diversity, polarity, and composition of cardenolides in their leaves, as well as in leaf toughness (SLM) and latex exudation (Table 3.1, PERMANOVA for composition $F_{2,160}$=131.51, P<0.001). However, we observed no influence of AMF inoculum availability on any of these chemical or physical resistance traits (Table 3.1, PERMANOVA for cardenolide composition: AMF $F_{2,160}$=1.62, P=0.128).

In contrast to their consistent effects on foliar cardenolide concentrations, AMF altered plant growth and nutritive traits in a plant species-specific fashion (Table 3.1, Figs. 3.5a,b). AMF inoculation decreased the aboveground biomass of most milkweed species by 8 to 29%. The exception was *A. curassavica*, in which AMF inoculation increased aboveground biomass by an average of 28% (Plant species*AMF $F_{6,215}$=2.69, P=0.0155, Fig. 3.5a). AMF inoculation increased foliar P concentrations in *A. curassavica* and *A. latifolia* by an average of 25% and 16%, respectively, but decreased P concentrations in *A. incarnata* and *A. syriaca* by an average of 8% and 13%, respectively (Plant species*AMF $F_{6,106}$=3.11, P=0.0076; Fig. 3.5b). In contrast,

AMF inoculum availability did not affect foliar C or N concentrations, or foliar C/N ratios, although these traits did vary among plant species (Table 3.1).

Discussion

Our study is among the first to document the impacts of AMF on toxin sequestration by specialist herbivores, while measuring simultaneously effects on herbivore performance. We demonstrate that 1) aphids and caterpillars sequester higher concentrations of cardenolides from plants inoculated with AMF, following AMF-mediated increases in foliar cardenolide concentrations. 2) AMF availability influences the performance of both aphids and caterpillars on milkweed, though in different ways. On all milkweed species, aphid performance varies nonlinearly with increasing AMF inoculum availability, with lowest performance under medium levels of inoculum availability and highest performance under high inoculum availability. In contrast, while caterpillar survival varies markedly with AMF inoculum availability, it does so in a plant species-specific manner, and caterpillar growth is unaffected by AMF. Our findings suggest that by altering plant phenotype, the availability of AMF in soil has the potential to influence both the top-down (via sequestration) and the bottom up (via plant defense and nutrition) forces that operate on milkweed herbivores.

Inoculation of plants with medium or high amounts of AMF inoculum resulted in equal levels of root colonization (Fig. B3.1). Nonetheless, we observed that the availability of AMF inoculum (medium versus high) influenced herbivore performance and plant phenotype (Tables 3.1,3.2). Because the commercial AMF mix that we used was purported to consist of four AMF species, the different effects of AMF availability on herbivore performance may be a function of differential colonization by AMF species under medium and high AMF availability. AMF species vary in their relative trading of nutrients (Lendenmann et al. 2011, Thonar et al. 2014, Argüello et al. 2016) and effects on plant phenotype (Gehring and Bennett 2009, Bennett et al. 2013) which can alter herbivore performance (Roger et al. 2013, Vannette and Hunter 2013). However, cloning and sequencing of the AMF mix, and roots from milkweed plants grown under the same experimental conditions, with AMF-specific primers (Krüger et al. 2009) demonstrated that the AMF mix consisted only of *Funneliformis mosseae* (details in Appendix B).

Instead, the differential effects of medium and high AMF inoculum availability on herbivore performance and plant phenotype are more likely due to differential regulation of AMF colonization by plants under medium and high AMF availability. Although AMF colonization levels increase with increasing inoculum availability (Garrido et al. 2010, Vannette and Hunter 2011), plants maintain a maximum level of AMF colonization of roots (Vierheilig et al. 2000a, Vierheilig et al. 2000b, Meixner et al. 2005) and suppress further colonization after reaching a critical level (Vierheilig 2004). Plant regulation of AMF development in roots is controlled by the same plant hormones (Staehelin et al. 2011, Gutjahr 2014, Bucher et al. 2014, and references therein) that are integral to the development of plant vascular tissues (Lucas et al. 2013) and the resistance responses of plants to insect herbivores (Pieterse et al. 2012, Pieterse et al. 2014). In our medium AMF treatment, there may have been sufficient inoculum to attain maximum levels of AMF colonization of plant roots. Therefore, under high AMF availability, plants may have suppressed AMF development in roots more strongly by altering phytohormone levels, resulting in the observed differences in herbivore performance and plant phenotype between medium and high AMF treatments.

Sequestration by specialist herbivores is altered by AMF availability

Both aphids and caterpillars sequestered higher concentrations of cardenolides when feeding upon plants under medium and high AMF inoculum availability (Figs. 3.1a,b), following AMF-mediated increases in foliar cardenolide concentrations (Figs. 3.4, B3.2a,b; Table B3.3). This is consistent with previous reports of tight links between aphid and caterpillar sequestration and host plant cardenolide concentrations (Malcolm 1990, Malcolm 1994, Agrawal et al. 2015, Petschenka and Agrawal 2015). However, while AMF inoculum availability did not influence the composition of cardenolides in foliage, AMF did affect the composition of cardenolides sequestered by aphids and caterpillars. Sequestration of cardenolides by *A. nerii* occurs through passive diffusion (Malcolm 1990, Züst and Agrawal 2016b). Therefore, AMF-mediated changes in the composition of cardenolides sequestered by aphids may result from AMF changing the relative concentrations of cardenolides present in phloem, but not leaves. While milkweed phloem contains the same variety of cardenolides as leaves, the concentrations of specific cardenolides may vary between phloem and leaves (Züst and Agrawal 2016b).

In contrast, monarch caterpillars may control the uptake of particular cardenolides and their amounts (Malcolm 1994, Tao and Hunter 2015) by sequestering cardenolides actively and selectively (Malcolm and Brower 1989, Frick and Wink 1995, Petschenka and Agrawal 2015, Erb and Robert 2016). AMF may have affected the composition of cardenolides sequestered by caterpillars, without affecting the composition of foliar cardenolides, by altering aspects of plant quality that may affect active sequestration, such as nutrient availability. We did not find correlations between foliar nutrient content and sequestration, potentially due to low sample sizes, but variation in soil N and P availability has been found to alter the efficiency of monarch caterpillar sequestration and the composition of cardenolides that monarch caterpillars sequester (Tao and Hunter 2015). Alternatively, interactions between AMF and caterpillar feeding may have altered the composition of foliar cardenolides (Bennett et al. 2009, Agrawal et al. 2014, Wang et al. 2015), resulting in the observed, AMF-mediated differences in caterpillar sequestration. However, milkweed responses to monarch caterpillar feeding can take up to five days to occur (Agrawal et al. 2014) and monarch caterpillars fed on our experimental plants for only six days. Therefore, we think it unlikely that AMF-mediated changes in caterpillar sequestration were driven by interactions between AMF and caterpillar induction of foliar cardenolides.

AMF abundance alters specialist herbivore performance and survival

The availability of AMF inoculum had consistent, nonlinear effects on aphid performance, regardless of milkweed species (Fig. 3.2). Aphids had the lowest per capita growth rates and individual masses on plants under medium AMF availability, yet had the highest per capita growth rates and masses on plants under high AMF availability (Fig. 3.2). Thus, we found within a single study the range of aphid responses to AMF from the literature, from positive to negative (Pacovsky et al. 1985, Gange and West 1994, Gange et al. 1999, Gange et al. 2002, Gehring and Whitham 2002, Wurst et al. 2004, Hempel et al. 2009, Koricheva et al. 2009, Abdelkarim et al. 2011, Babikova et al. 2014a, Colella et al. 2014, Williams et al. 2014, Grabmaier et al. 2014, Bennett et al. 2016, Simon et al. 2017, Tomczak and Müller 2017). Our findings suggest that some of the previously found variation in aphid responses may result from differences in AMF inoculum availability among studies.

AMF may have affected aphid performance by altering foliar cardenolide concentrations; we found that aphid masses declined with increasing foliar cardenolide concentrations (Table B3.3, Fig. B3.2d). Indeed, aphids had lower masses and per capita growth rates on plants under medium AMF availability (Fig. 3.2), which had greater foliar cardenolide concentrations than plants without AMF (Fig. 3.4). Although *A. nerii* tolerate cardenolides, they are negatively affected by high cardenolide concentrations (Agrawal 2004, de Roode et al. 2011, Birnbaum et al. 2017). Nonetheless, we interpret the regressions with caution due to low sample sizes and plant species-specific differences in traits. AMF-mediated increases in aphid performance under high AMF availability may also be a consequence of increased vascular bundle size; AMF colonization increases the size of vascular bundles in plants (Krishna et al. 1981), increasing aphid phloem feeding and reproductive success (Simon et al. 2017). Although aphids are often responsive to changes in amino acid content of phloem (Züst and Agrawal 2016a), we think it unlikely that AMF influenced *A. nerii* performance by changing phloem soluble sugar or amino acid content because previous studies found no correlations among AMF-mediated changes in aphid performance and foliar or phloem nutrient content (Gange and West 1994, Hempel et al. 2009, Grabmaier et al. 2014).

Although AMF colonization of plants has been found to increase the survival of specialist caterpillars (Goverde et al. 2000), we found that AMF inoculum availability improved, did not affect, or reduced the survival of a specialist caterpillar, depending on the plant species and density of AMF inoculum available to the plant (Fig. 3.3). This breadth of responses of monarch caterpillars to AMF among plant species may result from plant species-specific effects of AMF on plant biomass (Fig. 3.5a); caterpillar survival increased with increasing aboveground biomass (Table B3.3, Fig. B3.2e). Although caterpillars were never food limited in our study, AMF-mediated declines in plant biomass may have reduced caterpillar survival by decreasing the availability of young leaves because monarch caterpillars prefer younger leaves (Bingham and Agrawal 2010). AMF-mediated increases in foliar cardenolide concentrations did not correlate with declines in caterpillar survival in this study, although high cardenolide concentrations often reduce monarch caterpillar performance and survival (Zalucki et al. 2001a, Agrawal 2005, Rasmann et al. 2009, Tao et al. 2016b).

Interestingly, despite finding strong effects of AMF on monarch survival, we found no influence of AMF on monarch caterpillar growth rates (Table 3.2). Our findings confirm those for other specialist chewers, such as specialist beetle larvae and adult weevils (Cosme et al. 2011, Laird and Addicott 2008), whose growth rates are also unaffected by AMF. However, our findings contrast with previous work that found monarch caterpillar growth rates to increase on milkweed plants under higher AMF inoculum availability (Vannette and Hunter 2013). These conflicting findings may result from experimental milkweed plants being inoculated with different AMF species; individual AMF taxa and mixes alter plant phenotype differently (Bennett et al. 2009, Vannette and Hunter 2011), affecting caterpillar performance (Goverde et al. 2000, Roger et al. 2013). Indeed, AMF-mediated increases in monarch caterpillar growth rates were attributed to AMF-mediated declines in milkweed leaf toughness (SLM) and latex exudation (Vannette and Hunter 2013) and we found no influence of AMF on these traits (Table 1). In addition, it is possible that our plants were already induced by thrip activity, whereas plants in previous studies were not. However, because plants of all treatments were attacked equally, we do not believe that the minor thrip damage altered the quality of our results.

Effects of AMF on herbivore performance and toxin sequestration may have community-wide consequences

Because the availability of AMF inoculum altered both toxin sequestration and performance of specialist herbivores, AMF may affect herbivore populations by altering both top-down and bottom-up factors. For instance, aphids that fed upon milkweeds under medium AMF availability sequestered nearly twice the concentration of cardenolides that they did when feeding upon AMF-free plants, potentially improving aphid resistance to natural enemies. Aphid predators exhibit high rates of mortality when fed oleander aphids from high cardenolide milkweeds, but experience low rates of mortality when fed aphids from low cardenolide milkweeds (Malcolm 1992). Accordingly, in the field, oleander aphid populations are smaller and more influenced by predators when feeding on low cardenolide milkweed species than when feeding on high cardenolide milkweed species (Malcolm 1992, Mohl et al. 2016). Similarly, monarch caterpillars that sequester higher concentrations of cardenolides are more toxic to their predators (Brower et al. 1968, Reichstein et al. 1968, Brower and Moffitt 1974) and may be more resistant to their parasites (Lefèvre et al. 2010, Sternberg et al. 2012). Therefore, monarch

caterpillars may be better protected against their natural enemies when their host plants are inoculated with AMF.

The strong effects of AMF on aphid per capita growth rates and caterpillar survival suggest that the availability of AMF in soil may also influence the population dynamics of herbivores by changing host plant quality. Furthermore, by altering aphid densities and individual masses, AMF may influence aphid-parasitoid interactions. Parasitism rates of *A. nerii* are density dependent (Helms et al. 2004), and parasitoids that develop in larger herbivore hosts have larger clutch sizes, bigger individual offspring, greater proportions of female offspring, and increased longevity (Hunter 2003, Bukovinszky et al. 2008, van Veen and Godfray 2012). AMF colonization of plants has been found to increase parasitoid attack rates, shorten parasitoid developmental times, and increase successful emergence of aphid parasitoids (Hempel et al. 2009, Bennett et al. 2016), even in the absence of plant-derived cues such as volatiles (Bennett et al. 2016). Our study suggests that AMF-mediated increases in aphid size may be a simple mechanism by which AMF improve parasitoid success. In support of this, communities of other belowground organisms, such as soil-dwelling nematodes, have been found to improve parasitoid performance, potentially by increasing aphid size (Bezemer et al. 2005).

Conclusion

In summary, we found that AMF inoculum availability influences strongly toxin sequestration and performance of two specialist herbivores, suggesting that AMF availability may substantially alter interactions among plants, herbivores, and their natural enemies. Furthermore, the availability of AMF inoculum, measured as infectivity and spore abundances, varies on small scales, such as centimeters (Wolfe et al. 2007) and meters (Carvalho et al. 2003). Therefore, plants within a single population may experience substantial variation in AMF availability in soils. This variation in AMF abundance may result in spatial variation in plant quality for herbivores, and herbivore quality for their natural enemies, ultimately affecting large scale population dynamics (Riolo et al. 2015). Future studies should consider how natural AMF abundances influence plant phenotype and the resulting herbivore and natural enemy population dynamics in the field.

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Table 3.1. Effects of plant species, arbuscular mycorrhizal fungi (AMF) inoculum availability, and their interaction on plant traits, including the proportion of roots colonized by AMF, natural log-transformed foliar cardenolide concentrations, foliar cardenolide diversity, foliar cardenolide polarity, leaf toughness (specific leaf mass, SLM; mg/cm^2), natural log-transformed latex exudation (mg), aboveground biomass (mg), foliar P concentration (%), foliar C concentration (%), foliar N concentration (%), foliar C/N ratio. Numbers represent F-values and P-values from general linear mixed models. Final sample sizes per treatment are presented in Table B3.2 (see text for details). Note that because plants that received no experimental AMF inoculum remained free of AMF contamination, they were excluded from subsequent analyses of AMF colonization. Similarly, *A. incarnata* produced no foliar cardenolides in this study, and were therefore excluded from analyses of foliar cardenolides.

	Plant species		**AMF**		**Plant species * AMF**	
	F	***P***	***F***	***P***	***F***	***P***
Proportion AMF colonization	$F_{3,70}$=4.00	0.011**	$F_{1,70}$=0.56	0.4586	$F_{3,70}$=0.61	0.6122
Foliar cardenolide concentration	$F_{2,163}$=251.05	<.0001***	$F_{2,163}$=2.98	0.0538*	$F_{4,163}$=0.46	0.764
Foliar cardenolide diversity	$F_{2,157}$=351.18	<.0001***	$F_{2,157}$=1.51	0.2242	$F_{4,157}$=0.82	0.5147
Foliar cardenolide polarity	$F_{2,157}$=18.75	<.0001***	$F_{2,157}$=0.39	0.6779	$F_{4,157}$=0.84	0.5025
Leaf toughness (SLM)	$F_{3,221}$=113.58	<.0001***	$F_{2,221}$=0.37	0.691	$F_{6,221}$=1.36	0.2324
Latex exudation	$F_{3,218}$=79.24	<.0001***	$F_{2,218}$=0.62	0.5381	$F_{6,218}$=1.4	0.2167
Aboveground biomass	$F_{3,215}$=47.1	<.0001***	$F_{2,215}$=1.8	0.1681	$F_{6,215}$=2.69	0.0155**
Foliar P concentration	$F_{3,106}$=12.57	<.0001***	$F_{2,106}$=1.04	0.3556	$F_{6,106}$=3.11	0.0076**
Foliar C concentration	$F_{3,106}$=4.17	0.0078**	$F_{2,106}$=0.90	0.4112	$F_{6,106}$=1.04	0.4067
Foliar N concentration	$F_{3,106}$=9.24	<.0001***	$F_{2,106}$=0.34	0.7141	$F_{6,106}$=0.39	0.8866
Foliar C/N ratio	$F_{3,106}$=12	<.0001***	$F_{2,106}$=0.05	0.9535	$F_{6,106}$=0.16	0.9864

***P<0.001, **P<0.05, *P<0.1

Table 3.2. Effects of plant species, arbuscular mycorrhizal fungi (AMF) inoculum availability, and their interaction on measures of herbivore toxin sequestration and performance, including natural log-transformed cardenolide concentration sequestered by aphids (mg/g dry mass), diversity of cardenolides sequestered by aphids, natural log-transformed polarity of cardenolides sequestered by aphids, natural log-transformed cardenolide concentration sequestered by caterpillars (mg/g dry mass), diversity of cardenolides sequestered by caterpillars, natural log-transformed polarity of cardenolides sequestered by caterpillars, aphid per capita growth rate (r), individual aphid dry mass (mg), caterpillar growth rate, log-transformed caterpillar efficiency of conversion (ECI) of ingested biomass, and log-transformed leaf area consumed (CLA) by caterpillars. Numbers represent F-values and P-values from general linear mixed models. Final samples sizes are presented in Table B3.2 (see text for details) No aphid populations and few caterpillars sequestered cardenolides when feeding upon *A. incarnata*, so herbivores that fed upon *A. incarnata* were excluded from analyses of cardenolide sequestration.

	Plant species		**AMF**		**Plant species * AMF**	
	F	***P***	***F***	***P***	***F***	***P***
Toxin sequestration						
Aphid cardenolide concentration	$F_{2,48}$=9.24	0.0004***	$F_{2,48}$=3.35	0.0434*	$F_{4,48}$=0.08	0.9879
Aphid cardenolide diversity	$F_{2,48}$=23.48	<.0001***	$F_{2,48}$=0.01	0.9868	$F_{4,48}$=1.2	0.3221
Aphid cardenolide polarity	$F_{2,48}$=322.66	<.0001***	$F_{2,48}$=0.1	0.9028	$F_{4,48}$=2.12	0.0934
Caterpillar cardenolide concentration	$F_{2,100}$=35.76	<.0001***	$F_{2,100}$=4.05	0.0203*	$F_{4,100}$=1.93	0.1107
Caterpillar cardenolide diversity	$F_{2,100}$=36.49	<.0001***	$F_{2,100}$=4.07	0.02*	$F_{4,100}$=0.93	0.4488
Caterpillar cardenolide polarity	$F_{2,100}$=351.27	<.0001***	$F_{2,100}$=1.63	0.2016	$F_{4,100}$=2.96	0.0234*
Performance						
Aphid r	$F_{3,166}$=9.10	<.0001***	$F_{2,166}$=13.09	<.0001***	$F_{6,166}$=0.49	0.8154
Aphid individual mass	$F_{3,159}$=28.62	<.0001***	$F_{2,159}$=8.74	0.0003***	$F_{6,159}$=1.31	0.2536
Caterpillar growth rate	$F_{3,145}$=8.18	<.0001***	$F_{2,145}$=0.18	0.8343	$F_{6,145}$=0.26	0.9539
Caterpillar ECI	$F_{3,137}$=20.13	<.0001***	$F_{2,137}$=1.12	0.3284	$F_{6,137}$=1.62	0.1448
Caterpillar CLA	$F_{3,137}$=6.06	0.0007***	$F_{2,137}$=0.88	0.4154	$F_{6,137}$=1.1	0.3675

*** P<0.001; **P<0.01, *P<0.05

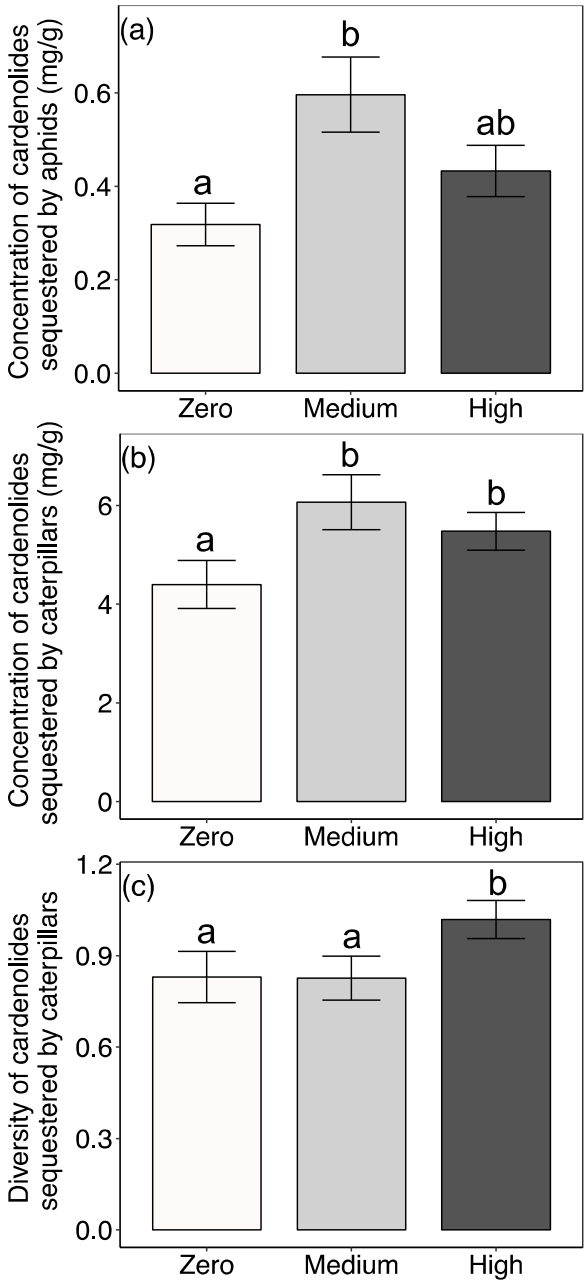

Figure 3.1. Effects of AMF inoculum availability on the concentration of cardenolides sequestered by a) aphid populations and b) individual caterpillars, and on c) the diversity of cardenolides sequestered by individual caterpillars. Sample sizes range from 15-24 aphid populations (= replicate plants) for aphid cardenolide concentrations, 39-43 individual caterpillars (= replicate plants) for the concentration and diversity of cardenolides sequestered by caterpillars per AMF treatment. Bars display the mean ± 1SE. Different letters indicate significantly ($P<0.05$) different means (Tukey post-hoc test of the ANOVA).

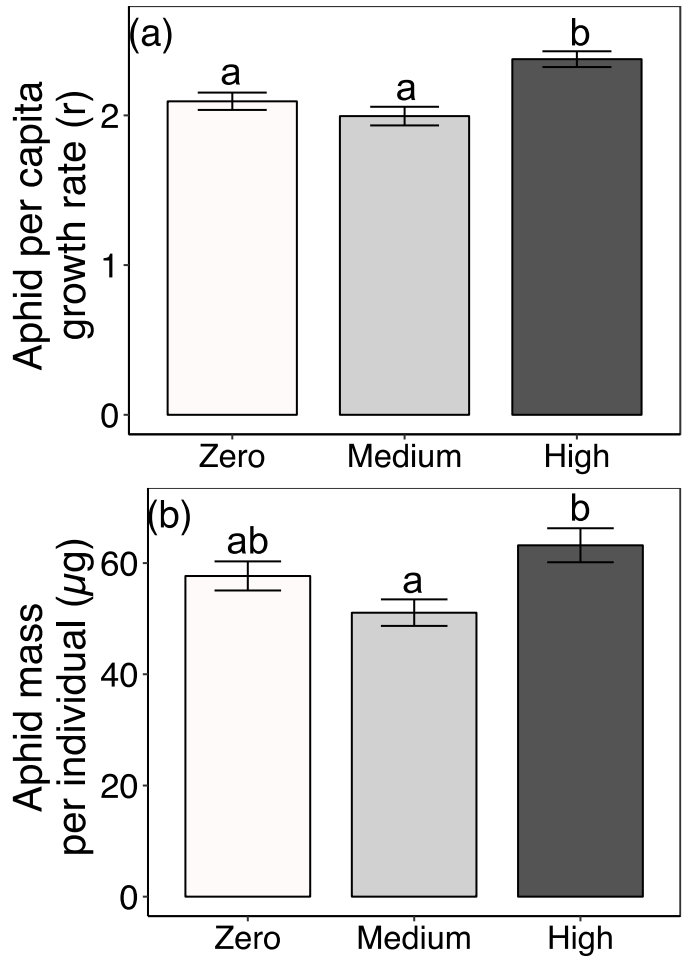

Figure 3.2. Effects of AMF inoculum availability on a) per capita growth rates of aphids (r over 6 days) and b) average dry mass of individual aphids reared on four milkweed species. Sample sizes are 60 populations of aphids (= replicate plants) for aphid per capita growth rates and range from 55-59 populations for average individual aphid mass per AMF treatment. Bars display the mean ± 1SE. Different letters indicate significantly ($P<0.05$) different means (Tukey post-hoc test of the ANOVA).

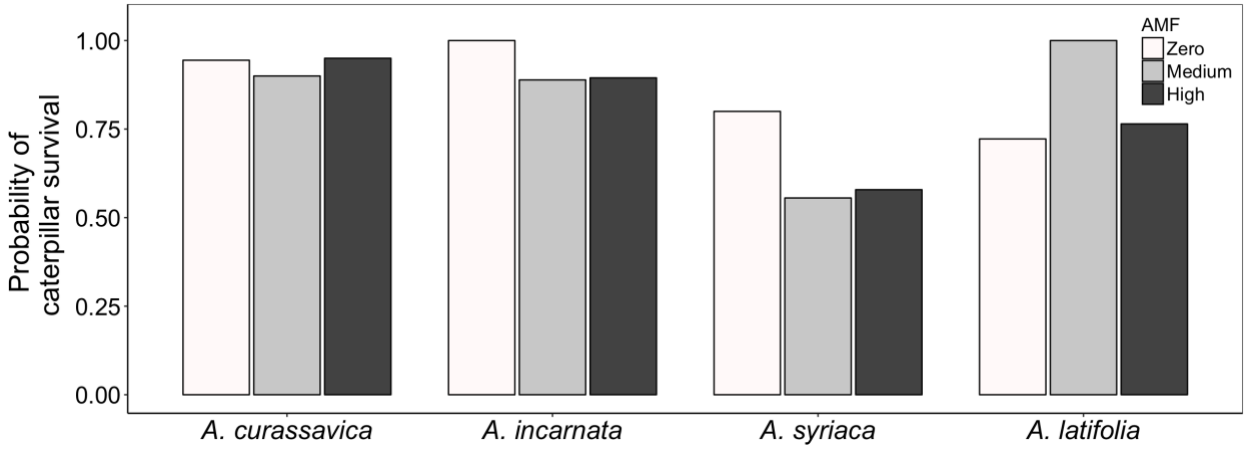

Figure 3.3. Effect of AMF inoculum availability on the probability of caterpillar survival on four milkweed species. Sample sizes range from 17-20 caterpillars (= replicate plants) per plant species x AMF treatment. Bars display the mean ± 1SE.

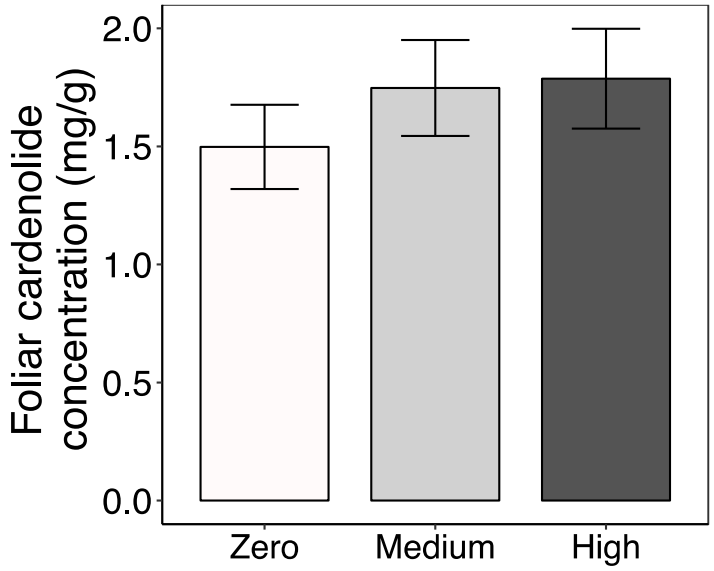

Figure 3.4. Effects of AMF inoculation on foliar cardenolide concentrations of three milkweed species. Samples sizes range from 58 to 59 plants per AMF treatment. Bars display the mean ± 1SE. Foliar cardenolide concentrations vary among AMF treatments ($P = 0.0538$), but treatment means are not significantly different at $P < 0.05$ (Tukey post-hoc test of the ANOVA).

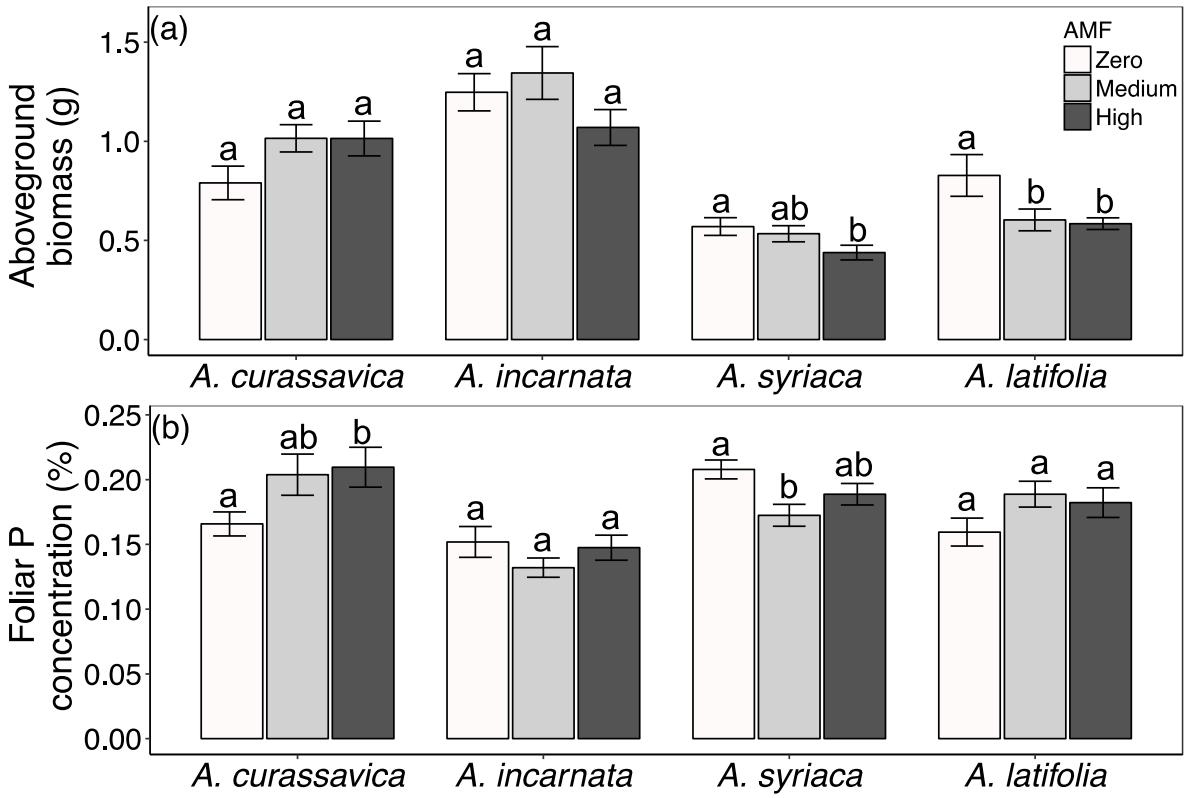

Figure 3.5 Effects of AMF inoculum availability on a) aboveground biomass and (b) foliar phosphorus (P) concentrations of four milkweed species. Sample sizes range from 17-20 plants per treatment for aboveground biomass and 9–10 plants per treatment for P concentrations. Bars display the mean ± 1SE. Different letters indicate significantly ($P < 0.1$) different AMF treatment means within each plant species (Tukey post-hoc test of the ANOVA within plant species).

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Chapter IV

Mycorrhizae alter constitutive and herbivore-induced volatile emissions by milkweeds

Abstract

Plants communicate with other members of their communities with volatile organic compounds (VOCs). Using VOCs, plants can cue natural enemies to their herbivore prey on plants, reducing damage by herbivores. However, herbivores also utilize volatile cues to identify appropriate host plants. Plant volatile emissions vary with biotic and abiotic conditions, generating substantial spatial and temporal variation in multitrophic interactions. Despite extensive efforts to understand sources of variation in plant communication by VOCs, we still lack an understanding of how ubiquitous mutualists of plants belowground, such as arbuscular mycorrhizal fungi (AMF), influence plant VOC emissions. In a full factorial experiment, we subjected plants of two milkweed species (*Asclepias incarnata* and *A. curassavica*) under three levels of AMF inoculum availability to damage by aphids (*Aphis nerii*) or no herbivores for seven days. We then measured plant headspace volatiles, foliar cardenolides (chemical defenses), and plant biomass.

We found that AMF have strong, plant-species specific effects on constitutive and aphid-induced VOC emissions. High AMF availability increased emissions of total VOCs, green leafy volatiles, and methyl salicylate in *A. curassavica* but decreased emissions in *A. incarnata*. In contrast, aphids consistently increased emissions of 6-methyl-5-hepten-2-one and benzeneacetaldehyde in both *A. incarnata* and *A. curassavica*, but AMF did not affect these emissions. Aphids suppressed emissions of individual terpenes (cis-ocimene, copaene, beta-cubebene, sesquiterpene 2, delta-cadinene) in the absence of AMF. However, high AMF availability suppressed terpene emissions to levels equivalent to those mediated by aphids, such that aphid damage on plants under high AMF availability did not further suppress terpene emissions. Lastly, aphid feeding suppressed cardenolide concentrations only in *A. curassavica*,

and AMF did not affect cardenolides in either plant species, despite having strong effects on VOC emissions. Our findings suggest that by altering milkweed VOC profiles, AMF may generate subsequent effects on herbivore and natural enemy attraction, and that AMF may affect the indirect defenses of these milkweed species differently.

Introduction

Plants communicate with and respond to other members of their communities via volatile organic compounds (VOCs) (Kessler and Baldwin 2001, Dicke and Baldwin 2010, Hare 2011, Karban et al. 2014b, Rowen and Kaplan 2016, Turlings and Erb 2018). Using VOCs, plants communicate not only among distant plant parts (Frost et al. 2007, Heil and Ton 2008), but also with neighboring plants (Karban et al. 2014a, 2014b, 2016). Furthermore, plant signals cross species boundaries and trophic levels, cuing natural enemies to their herbivore prey on plants, and reducing damage by herbivores (Kessler and Baldwin 2001, Turlings and Erb 2018). Simultaneously, herbivores utilize volatile cues to identify appropriate hosts (Bruce et al. 2005, Bruce and Pickett 2011). Plant volatile emissions vary with plant genotype, the identity of the herbivore attackers, and abiotic conditions, generating substantial spatial and temporal variation in multitrophic interactions (Gouinguene 2002, van Poecke 2002, Loreto et al. 2006, Staudt et al. 2010, Holopainen and Gershenzon 2010, Trowbridge et al. 2014, Rowen and Kaplan 2016, Turlings and Erb 2018).

Despite extensive efforts to understand sources of variation in plant communication by VOCs (Turlings and Erb 2018), we still lack an understanding of how ubiquitous mutualists of plants, such as arbuscular mycorrhizal fungi (AMF), influence plant constitutive and herbivore-induced VOC emissions. AMF colonize the roots of over 80 percent of plant species, providing nutrients and water in exchange for plant sugars (Wang and Qiu 2006, Smith and Read 2008). In doing so, AMF interact with plant defensive signaling pathways, including the jasmonic acid (JA) and salicylic acid (SA) pathways (Jung et al. 2012, Cameron et al. 2013, Gutjahr 2014, Bucher et al. 2014). By altering plant nutrient uptake and defensive signaling pathways, AMF influence a diversity of plant primary and secondary metabolites (Bennett et al. 2009, Vannette et al. 2013, Roger et al. 2013, Schweiger et al. 2014, Schweiger and Müller 2015), affecting plant quality for herbivores (Hartley and Gange 2009, Koricheva et al. 2009). The association with

AMF is often mutualistic for plants; AMF frequently stimulate plant growth and mitigate abiotic and pathogen stress (Smith and Read 2008). However, the effects of AMF on plant growth and defense range from beneficial to detrimental, depending on the environment (Hoeksema et al. 2010), plant and AMF identity (Klironomos 2003, Tao et al. 2016), and the density of AMF inoculum available to plants (Garrido et al. 2010, Vannette and Hunter 2011, 2013). While the effects of AMF on constitutive and herbivore-induced direct defenses are well-established (Bennett et al. 2009, Kempel et al. 2010, Vannette and Hunter 2011, Barber 2013, Wang et al. 2015, Tao et al. 2016) the role of AMF in shaping plant constitutive and herbivore-induced volatile emissions remain far less understood.

The few studies that have assessed AMF effects on plant volatile emissions demonstrate that AMF have strong, but variable, effects on constitutive and herbivore-induced foliar volatile profiles. For instance, AMF increase (Asensio et al. 2012, Schausberger et al. 2012, Shrivastava et al. 2015), decrease (Fontana et al. 2009, Leitner et al. 2010, Babikova et al. 2014b, 2014a), or do not affect foliar terpene emissions (Rapparini et al. 2008), depending on the specific AMF and plant species involved. Similarly, AMF have species-specific effects on green leafy volatile (GLV) emissions (Fontana et al. 2009, Babikova et al. 2014a). These volatile classes are known to influence herbivore and natural enemy attraction (Bruce et al. 2005, Arimura et al. 2009, Turlings and Erb 2018), indicating that AMF mediation of VOC profiles may affect multitrophic interactions. Indeed, both aphids (Babikova et al. 2014b, 2014a) and their parasitoids (Guerrieri et al. 2004) are more attracted to plants colonized by AMF. Interestingly, mycorrhizal plants not damaged by aphids are as attractive to parasitoid wasps as are non-mycorrhizal plants infested with aphids (Guerrieri et al. 2004). Similarly, predatory mites (*Phytoseiulus persimilis*) are more attracted to volatiles produced by mycorrhizal plants infested with spider mites (*Tetranychus urticae*) than to those of infested plants without AMF (Schausberger et al. 2012).

Most studies to date assessing how AMF influence plant VOC emissions have been limited to crop plant species, and few have considered how AMF affect plant direct and indirect defenses simultaneously (but see Fontana et al. 2009). Furthermore, no study to date has considered how the availability of AMF inoculum in soil influences VOC emissions. The extent of AMF inoculum available to plants varies among habitats (Koide and Mooney 1987,

Soudzilovskaia et al. 2015) and with land management practices (Lekberg and Koide 2005). AMF availability also varies on small scales, such as meters (Carvalho et al. 2003) and centimeters (Wolfe et al. 2007). Plant constitutive (Vannette and Hunter 2011, Vannette et al. 2013, Tao et al. 2016) and herbivore-induced direct defenses (Meier and Hunter 2018b) are affected substantially by the availability of AMF, with consequences for insect herbivores (Vannette and Hunter 2013, Meier and Hunter 2018a). Therefore, it seems likely that the extent of AMF inoculum available to plants may also influence plant constitutive and induced VOC profiles.

Here, we evaluate how AMF influence constitutive and aphid-induced VOC emissions and direct chemical defenses in two closely related plant species. We performed a full-factorial experiment, manipulating oleander aphids (*Aphis nerii*) on two milkweed species (*Asclepias incarnata* and *A. curassavica*) provided with different amounts of AMF inoculum. Based on previous studies of *A. nerii* (Zehnder and Hunter 2007, de Roode et al. 2011), we expected aphid feeding to decrease plant direct and indirect defenses to varying extents between plant species. Furthermore, we expected AMF to alter both constitutive and aphid-induced VOC emissions in a plant species-specific manner, with the strength of these effects varying with AMF availability. Because the outcomes of AMF-plant associations on VOC emissions are specific to the AMF and plant species (above), we did not have specific predictions for the direction of these effects.

Methods

Study System

We used two milkweed species (*Asclepias incarnata* and *A. curassavica*) that vary substantially in their direct chemical defenses (cardenolides) and exhibit AMF-mediated variation in their cardenolide concentrations (Vannette et al. 2013, Tao et al. 2016). Cardenolides are toxic, bitter-tasting steroids that disrupt the functioning of sodium-potassium channels in animal cells (Agrawal et al. 2012) and negatively affect specialist milkweed herbivores, despite herbivore adaptations to resist these defenses (Agrawal 2004, Sternberg et al. 2012). Although no study to date has considered how aphid feeding affects VOC emissions in milkweeds, milkweed species vary in their sesquiterpene emissions, which correlate with top-down pressure by predators on herbivores in the field (Mooney et al. 2010). Furthermore, common milkweed

plants (*A. syriaca*) emit herbivore-induced plant volatiles (HIPVs) in response to caterpillar damage that attract natural enemies, indicating that milkweed species have effective indirect defenses (Wason and Hunter 2014). Feeding by oleander aphids (*A. nerii*) suppresses cardenolide concentrations in milkweeds (Zehnder and Hunter 2007, de Roode et al. 2011), and the extent of suppression varies with AMF availability (Meier and Hunter 2018b). Therefore, we hypothesized that AMF and aphids may also interact to affect milkweed indirect defenses. In the field, *Aphis nerii* are killed by a suite of generalist predators and parasitoids, including lacewings (Neuroptera), syrphids (Diptera), coccinelids (Coleoptera), spiders (Araneae), aphid midge flies (*Aphidoletes aphidimyza*; Diptera), and parasitoid wasps (Hymenoptera) (Malcolm 1992, Helms et al. 2004, Mooney et al. 2010, Mohl et al. 2016). As these predators and parasitoids respond to HIPVs in other systems (Dicke and Baldwin 2010, Turlings and Erb 2018), it is likely that AMF and aphid-mediated changes in VOC emissions in milkweeds could affect natural enemy attraction in the field.

Asclepias incarnata seeds were collected from naturally occurring populations in Emmet County, MI. *Asclepias curassavica* seeds were purchased from Victory Seeds (Mollala, OR, www.victoryseeds.com). We used AMF inoculum from Mycorrhizal Applications (Grants Pass, OR, USA), which is advertised to contain four AMF species including *Rhizophagus intraradices*, *Funneliformis mosseae*, *Glomus aggregatum*, and *Claroideoglomus etunicatum* (33 spores of each AMF species per gram inoculum, www.plant-success.com). However, we recently found this mix to contain only *Funneliformis mosseae* (Meier and Hunter 2018a). Milkweed species grow in habitats that host a diversity of AMF taxa (Öpik et al. 2006), and can form associations with these cosmopolitan AMF species in natural and experimental populations (Vannette et al. 2013, Tao et al. 2016, Meier and Hunter 2018a, 2018b), although the frequency of such interactions is unknown. Oleander aphids were derived from a single aphid collected in October 2016 from Ann Arbor, MI and reared indoors on *A. tuberosa* for one month prior to the experiment.

Experimental protocols

After 6 weeks of cold, moist stratification at 4 ºC, we surface-sterilized seeds in 5% bleach, and then germinated them at room temperature (*A. curassavica* did not require

stratification). We planted seedlings in a mix of autoclaved soil (Metromix 360 Metro-Mix 380; MetroMix Sun Gro Horticulture Canada CM Ltd., Vancouver, BC, Canada) and sand (5:3) with AMF inoculum in conical deepots (D40H, Steuwe and Sons Inc., Corvalis, OR, USA). We manipulated the amount of AMF inoculum available to plants to generate zero, medium, and high levels of AMF colonization, which is possible because the amount of inoculum available to milkweed plants alters the proportion of roots colonized by AMF (Vannette and Hunter 2011, 2013, Tao et al. 2016). We homogenized 4.20 g live AMF inoculum (high treatment), 1.20 g live and 3.00 g autoclaved inoculum (medium treatment), or 4.2 g autoclaved inoculum (zero treatment) in 200 ml autoclaved soil and sand. The inoculum was placed between 400 ml of autoclaved soil and sand to prevent the transfer of AMF spores and hyphae among experimental plants. We returned the natural bacterial community of the potting soil to each pot by adding 20 ml of a bacterial solution made by filtering a suspension of 100 ml potting soil in 1L deionized water through an ultra-fine sieve (32 micron) to remove AMF hyphae and spores. Plants were fertilized weekly with 30 ml of 15-0-15 (N-P-K, 567 ppm) dark weather fertilizer (JR Peters Inc., Allentown, PA) and watered *ad libitum*. All experiments were conducted in a greenhouse under a 15L:9D regime. To not confound plant age with VOC measurements, we planted one set of plants in May 2017 and another three weeks later in June 2017.

Volatile collections

In a fully factorial design, we placed 20 reproductive oleander aphids or no herbivores on 12 plants of each plant species x AMF treatment (n=144). We allowed aphids to feed and reproduce for seven days to attain population sizes representative of those we have observed in the field (Helms et al. 2004). Dead or missing reproductive aphids were replaced for the first three days. Seven days of feeding is sufficient for oleander aphids to induce milkweed direct defenses in these milkweed species (Meier and Hunter 2018b), and in other systems is sufficient for aphids to induce plant volatiles (Kunert et al. 2002, Schaub et al. 2010). All plants were enclosed in white nylon mesh bags to prevent aphid movement among experimental plants. Nets were secured around the plant stem over a strip of cotton to prevent damage to the stem.

After seven days of aphid feeding, or no damage, we collected plant volatiles once from each individual plant using a pull-system. Aphids were allowed to remain on plants during VOC

collections, as this represents the most ecologically relevant combination of VOCs for natural enemies. Our sampling system allowed us to collect VOCs from seven chambers each day (6 experimental plants, 1 empty control chamber). Because we were only able to collect VOCs from six experimental plants per day, we could only measure VOCs from experimental plants of one plant species, with all AMF x aphid treatment combinations represented, per day. We therefore measured volatiles from each plant species every two days, with the species order randomized, to avoid confounding plant species with sampling date. In order to collect volatiles from all 144 plants, we collected VOCs from July 11 through August 3, 2017, establishing aphids on their experimental plants seven days before measurement. Despite planting over two dates, plants varied between 6 and 9 weeks of age when sampled for volatiles, with plant ages equally represented among all treatments. On each sampling day, only plants from the same planting date were chosen. All plants lacked reproductive structures when sampled.

Plants were enclosed in 9 L glass chambers placed atop teflon guillotines (Sigma Scientific LLC, Micanopy, FL, USA), which separated plant roots and potting soil from the volatile collections. Cotton strips were wrapped around the base of the plant stem where it entered the guillotine plate to prevent aphids from leaving the chamber. Aluminum foil was wrapped around the root collar at the top of each pot to further prevent root and soil volatiles from entering the chamber. After placing plants in the chambers, we first withdrew air from the enclosures for 1 h through teflon tubing with chemical traps bypassed to purge any volatiles released due to handling of the plants. Flowmeters and a vacuum pump (CADS-8Pull System, Sigma Scientific LLC, Micanopy, FL, USA) maintained the flow rate for each enclosure at 1.3 L min^{-1} for both the purge and sample collections. After the purge, we placed volatile collection traps containing 25 mg of Porapak Q (Sigma Scientific LLC, Micanopy, FL, USA) inline at the top of the chambers secured in teflon corks. To control for ambient volatiles, we also collected VOCs from an empty chamber on each sampling day. We collected volatiles for 8 h from approximately 11:00 to 19:00 each day. To prevent overheating in the greenhouse, we partially shaded the entire setup using shade cloth. Chambers and guillotine plates were rinsed with hexane after removing plants each evening, and again before enclosing new experimental plants the following morning to ensure that there were no residual volatiles from the previous day of sampling.

Immediately after sampling, we removed volatile collection traps, wrapped them in aluminum foil, and kept them on ice until returning them to the lab within an hour. We counted aphids present on each plant, rinsed aphids and honeydew off plants with RO water, and thoroughly cleaned roots in RO water. One leaf of the third leaf pair was taken from each plant, dried at 50 ºC, weighed, and then stored in methanol at -10 ºC until cardenolide analysis. Above and belowground tissues were dried in paper bags at 50 ºC and weighed to measure above and belowground biomass. A random subsample of approximately 20 mg of dry fine root was taken from each plant, rehydrated for 48 h, and stained to quantify AMF colonization. Specifically, roots were cleared with 10% KOH for 10 min, acidified using 2% HCl, and stained in 0.05% trypan blue in 1:1:1 water: glycerol: lactic acid (Vannette and Hunter 2011). We mounted roots on slides and scored AMF colonization in at least 100 root intersections per plant using the magnified gridline intersect method (McGonigle et al. 1990) with a Nikon compound microscope (Melville, NY, USA). An intersection was considered colonized if AMF hyphae, arbuscules, spores, or vesicles were present.

To account for effects of environmental variables on VOC emissions (Kesselmeier and Staudt 1999, Loreto and Schnitzler 2010), we measured temperature and relative humidity using HOBO data loggers (Onset Computer Corporation, Bourne, MA, USA) and photosynthetically active radiation using PAR sensors (SQ-420, Apogee Instruments, North Logan, UT, USA) every minute over the 8 hours of sampling. Three sensors measuring temperature and relative humidity and two sensors measuring PAR were placed between the chambers. We summed the data from each sensor from each minute over the 8 sampling hours to yield a cumulative value for each of the variables per sampling day. We combined the factors using principal components analysis (PCA) with the package FactoMineR (Lê et al. 2008) to account for covariance among environmental variables. PCA axes were kept if the eigenvalue of the axis was greater than one and the axis explained at least 10% of the total variance in the PCA. Ultimately, environmental data were combined into two PCA axes which explained 58.2 and 31.4% of the daily variation in temperature, humidity, and PAR. These PCA axes were used as covariates in all analyses of VOCs to account for variation in VOC emissions with environmental variables.

Analysis and identification of volatile organic compounds

We eluted volatile collection traps with 250 µl n-hexane (MS SupraSolv, Sigma-Aldrich) and purged traps with N_2 to ensure that all hexane was eluted. We added 0.161 ng nonyl acetate to 50 µl of each sample as an internal standard, while keeping all samples on dry ice to prevent evaporation. Samples were stored at -70 ºC until analysis. We analyzed 2-ul aliquots of VOC samples by gas chromatography mass spectrometry (GCMS, Agilent Technologies, Santa Clara, CA, USA) using the following GC method: injector held at 250 °C, initial column temperature at 50 °C held for 10 min, ramped at 5 °C min^{-1} to 200 °C, held for 10 min, with helium carrier gas at a flowrate of 1.3 ml min v. We used an Agilent J&W HP-5ms Ultra Inert 30 m x 0.25 mm inner-diameter column with 0.25 µm film thickness (Agilent Technologies, Santa Clara, CA, USA).

We tentatively identified compounds using the NIST 2005 library database. In addition, we injected a continuous series of n-alkanes (C_8-C_{24}; Sigma-Aldrich) to calculate linear retention indices for each compound on the same column used in the above analyses. We compared calculated retention indices of peaks from representative samples and standards to published values available on Pherobase (pherobase.com). When possible, we also verified the identity of peaks using authentic standards. Ultimately, we verified the identity of 32 out of 49 plant peaks, with 17 remaining unverified. When possible, we estimated the chemical classes of unverified peaks tentatively according to the number of carbons in the compounds and class proposed by the NIST database, and by the MS and peak retention times. Our estimates took into account the top three identifications proposed by the MS. We could not identify 13 compounds to chemical class because the MS did not provide a consistent molecular formula for those peaks.

We quantified the concentrations of each compound by comparing its peak area with that of the internal standard, nonyl acetate. Synthetic chemicals and any VOCs consistently collected in empty, control chambers were omitted from the dataset. To account for ambient concentrations of any additional plant volatiles, we subtracted the concentrations of compounds present in the control chamber from the concentrations measured in chambers containing experimental plants on the same sampling day. Concentrations of volatiles were standardized for

the number of hours for which they were collected from each chamber, and for aboveground plant biomass.

Direct defenses

To compare how AMF and aphids affect the direct chemical defenses of milkweed relative to their effects on the VOCs estimated above, we quantified foliar cardenolide concentrations following established methods (Zehnder and Hunter 2007, Meier and Hunter 2018a, 2018b). In brief, cardenolides were extracted from foliar samples in methanol. Samples were then separated by ultra performance liquid chromatography (UPLC; Waters Inc., Milford, MA, USA) using a Luna 2.5 μm C18(2) column (Phenomenex Inc., Torrance, CA, USA) with digitoxin as an internal standard. Peaks with symmetrical absorbance between 218 and 222 nm were quantified as cardenolides and total cardenolide concentrations were calculated as the sum of individual peaks. In addition, we calculated cardenolide diversity using Shannon's index and a cardenolide polarity index (the relative representation of lipophilic cardenolides) by summing the relative peak areas multiplied by each peaks' retention time (Rasmann and Agrawal 2011, Sternberg et al. 2012). Evidence suggests that more diverse and lipophilic cardenolides are more toxic than are less diverse or more polar mixes (Fordyce and Malcolm 2000, Zehnder and Hunter 2007, Sternberg et al. 2012).

Statistical Analyses

To compare the effects of AMF inoculum availability and aphid feeding on VOC emissions, we used linear mixed models using the lmer function in the lme4 package in R v 3.5.0. Significance of treatments was assessed using the anova function in the lmerTest package. Measures of VOCs were the dependent variables and milkweed species, AMF inoculum availability, aphid treatment, and their interactions were fixed effects. To account for variation in environmental factors during VOC sampling, we included the two PCA axes of environmental variables (see above) as covariates. In addition, we included the identity of the chamber in which the VOCs were collected and sampling date as random effects. We fit models for total VOC emissions, VOC compound classes, and individual compounds separately. The residuals of all analyses were checked for normality and homogeneity of variance. Total VOC emissions were log-transformed, and individual compounds and classes were cube root transformed. If one plant

species never produced a particular VOC, that species was excluded from analyses of that particular compound. We also evaluated differences in volatile community composition among plant species, AMF treatments, aphid feeding, and their interactions using permutational multivariate ANOVA (PERMANOVA; McCune et al. 2002). To do so, we used the adonis function in the vegan package (Oksanen et al. 2017) and calculated dissimilarities among samples using the Bray-Curtis metric for PERMANOVA.

To assess whether the final number of aphids on each plant during VOC sampling varied among plant species and AMF treatments, we fit a generalized linear mixed model with a Poisson distribution and log link function using the glmer function in the lme4 package. Aphid number was the dependent variable, plant species, AMF treatment, and their interaction were fixed effects, and sampling date was a random effect. Significance of treatments was assessed by Wald Chi Square analysis of deviance using the Anova function in the car package. In addition, to evaluate whether aphid density affected VOC emissions, we fit general linear mixed models with volatiles that were affected by the aphid feeding treatment (Table 4.2) as the dependent variables. Aphid density and PCA axes of environmental variables were covariates, and plant species, AMF treatment, and their interaction were fixed factors. Sampling date and chamber identity were designated as random effects. The residuals of analyses were checked for normality and homogeneity of variance.

In addition, we used linear mixed models to evaluate how AMF and aphids influenced foliar cardenolide concentration, diversity, and polarity, as well as the proportion of roots colonized by AMF, aboveground biomass, and belowground biomass. Plant traits were dependent variables while plant species, AMF inoculum availability, aphid treatment, and their interactions were fixed effects. Sampling date was a random effect. The residuals of all analyses were checked for normality and homogeneity of variance. Cardenolide concentrations and belowground biomass were natural log-transformed.

Results

AMF colonization

Inoculation of plants with AMF led to successful root colonization, such that plants inoculated without live AMF had no colonization (0 ± 0%), and inoculation with medium and high amounts of AMF resulted in an average of 4 (± 1.4) and 11 (± 1.7) percent root colonization, respectively (AMF $F_{2,110}$=23.5, P<0.001). Plants exposed to high AMF availability had 5.2 (± 1.0) percent arbuscules, plants exposed to medium AMF availability had 1.6 (± 0.9) percent arbuscules, and plants without AMF had no arbuscules ($F_{2,110}$=12.42, P<0.0001). The proportion of roots colonized by AMF did not vary between plants species and was unaffected by aphid feeding (Table 4.1).

VOC emissions

Asclepias incarnata and *A. curassavica* differed strongly in their VOC profiles. *Asclepias incarnata* plants emitted four times greater concentrations of volatiles than did *A. curassavica* plants ($F_{1,19.9}$=7.32, P=0.0136, Fig. 4.1). The composition of the volatile profiles also varied substantially between species (PERMANOVA $F_{1,132}$=25.78, P<0.001). *A. incarnata* plants emitted up to 48 different VOCs, whereas *A. curassavica* emitted up to 17 (Tables 4.2, 4.3). Most striking were the plant species-specific differences in sesquiterpenoid emissions; *A. incarnata* plants emitted up to 15 different sesquiterpenoids, which made up 11% of their total VOC emissions, whereas *A. curassavica* plants produced only 2 sesquiterpenoids, which made up only 2% of their total VOC emissions (Tables 4.2, 4.3).

Importantly, the amount of AMF available to plants altered total VOC emissions strongly. Inoculation with high amounts of AMF increased total VOC emissions in *A. curassavica* by 61%, but reduced VOC emissions in *A. incarnata* by 27% compared to plants under medium or zero AMF availability (Plant species*AMF: $F_{2,108.2}$=3.48, P=0.0343, Fig. 4.1). Aphid feeding did not affect total VOC emissions (Table 4.2). Furthermore, neither AMF nor aphids affected the composition of VOCs emitted, as estimated by PERMANOVA (AMF: $F_{2,132}$=0.498, P=0.859; Aphids: $F_{1,132}$=0.6268, P=0.613; AMF x Plant species $F_{2,132}$=1.3817, P=0.202; AMF x Aphids: $F_{2,132}$=0.771, P=0.61, Plant species x Aphids: $F_{1,132}$=0.4221, P=0.823, AMF x Plant species x Aphids: $F_{2,132}$=0.5491, P=0.833), despite affecting the concentrations of individual compounds (Tables 4.2, 4.3 and below).

AMF, but not aphids, had strong, plant species-specific effects on GLV and methyl salicylate emissions (Fig. 4.2, Tables 4.2, 4.3). *A. incarnata* plants emitted 40% less GLVs under high than medium AMF availability, while *A. curassavica* plants emitted 107% more GLVs under high AMF availability than under medium AMF availability. Plants without AMF produced intermediate levels of GLVs in both plant species (Plant species*AMF $F_{2,109}$=3.5, P=0.0336, Fig. 4.2a). This pattern was consistent for most GLVs detected, including 3-hexenyl-acetate, hexyl acetate, (2Z)-2-hexenyl acetate, and benzyl alcohol (Tables 4.2,4.3). Similarly, methyl salicylate emissions by *A. incarnata* decreased by 28% under high AMF availability, whereas methyl salicylate emissions increased by 76% in *A. curassavica* plants under high AMF availability, in comparison to plants under zero or medium AMF availability (Plant species*AMF $F_{2,107.6}$=3.31, P=0.0402, Fig. 4.2b). As methyl salicylate made up, on average, 88% of total benzenoid emissions in both plant species, AMF effects on methyl salicylate paralleled their effects on total benzenoid emissions (Plant species*AMF $F_{2,107.5}$=3.19, P=0.045). In addition, unknown compound 9 followed the same pattern as GLV and methyl salicylate emissions, such that inoculation of *A. incarnata* plants with high amounts of AMF reduced emissions by 48%, but in *A. curassavica*, high AMF availability increased emissions by 44% compared to plants without AMF (Plant species*AMF $F_{2,107.7}$=4.56, P=0.0126, Fig. 4.2c).

Aphid feeding did have distinct, plant species-specific effects on individual volatile compounds and classes. For example, aphids increased emissions of 6-methyl-5-hepten-2-one by 47% in both plant species (Aphids: $F_{1,110}$=6.15, 0.0147, Fig. 4.3a), driving the overall aphid-mediated increase in ketone emissions (Aphids: $F_{1,107.5}$=9.74, P=0.0023). In addition, aphid feeding increased benzeneacetaldehyde emissions by 3.3 times in *A. incarnata* and 8.7 times in *A. curassavica* (Aphids: $F_{1,107.1}$=116.1, P<0.001, Fig. 4.3b). AMF availability did not influence the emission of either compound (Table 4.2). The number of aphids present on plants during VOC collections varied slightly among AMF treatments between plant species, such that aphid abundances were greatest on *A. curassavica* plants under high AMF availability, but were greatest on *A. incarnata* plants under medium AMF availability (Plant species*AMF: χ^2=91.48, df=2, P<0.001, Table C4.1). Emissions of benzeneacetaldehyde increased with aphid abundance (Aphid abundance $F_{1,61.3}$=4.21, P=0.0446), but aphid abundance did not influence the emissions of any other compounds that were affected by our aphid feeding treatment (Table C4.2).

Importantly, the effects of aphid feeding on the emissions of individual terpenes varied with AMF availability, highlighting the role that AMF play in mediating VOC production in response to herbivore damage. For example, in the absence of AMF, aphids suppressed emissions of the monoterpene cis-ocimene by 70% and 72% in *A. incarnata* and *A. curassavica*, respectively (Fig. 4.4). Inoculation with medium or high amounts of AMF also suppressed emissions in *A. incarnata* and *A. curassavica* by 63% and 37%, respectively, in the absence of aphids. However, aphids could not suppress cis-ocimene emissions to the same degree under medium and high AMF availability that they could under zero AMF availability (AMF*Aphids: $F_{2,110}$=3.14, 0.0472, Fig. 4.4). Similarly, aphids suppressed the emissions of four sesquiterpenes produced by *A. incarnata* (copaene, beta-cubebene, delta-cadinene, and sesquiterpene 2), by 58 to 86% in those plants without AMF or under medium AMF availability (Fig. 4.5, Table 4.2). In the absence of aphids, high AMF availability suppressed emissions of these compounds by 60 to 79% compared to plants without AMF or under medium AMF availability (Fig. 4.5). Similar to the pattern for cis-ocimene, aphid suppression of these four sequiterpenes was compromised under high AMF (Fig. 4.5, Table 4.2), and aphids even increased emissions of sesquiterpene 2 by 2.7 times in *A. incarnata* plants under high AMF availability (Fig. 4.5c). Unknown compound 10 and caryophyllene emissions were affected in the same manner by aphids and AMF, although effects were only marginally significant for caryophyllene (Table 4.2, 4.3).

Direct defenses and biomass

As expected (Rasmann and Agrawal 2011, Vannette et al. 2013), foliar cardenolide concentrations differed greatly between milkweed species, with *A. curassavica* producing 33.9 times higher concentrations of cardenolides than *A. incarnata* (Plant species: $F_{1,22}$=616.08, P<0.001, Fig. 4.6). In addition, *A. curassavica* produced more diverse and lipophilic cardenolides, as well as an overall different composition of cardenolides than did *A. incarnata* (Diversity: $F_{1,21.2}$=2514.66, P<0.001; Polarity: $F_{1,21.8}$=6.49, P=0.0185; PERMANOVA $F_{1,125}$=137.75, P<0.001). Also as expected (de Roode et al. 2011), aphid feeding suppressed foliar cardenolide concentrations in *A. curassavica* by 14%, but did not affect cardenolide concentrations in *A. incarnata* (Plant species x Aphids: $F_{1,110}$=6.95, P=0.0096). In contrast to the strong effects of AMF on VOC emissions (above), AMF did not affect foliar cardenolide

concentrations. In addition, neither aphids nor AMF affected the diversity, polarity, or composition of cardenolides expressed in either plant species (Table 4.1, PERMANOVA AMF: $F_{2,125}$=1.303, P=0.222, Aphids: $F_{1,125}$=0.995, P=0.380)

Aphids reduced the aboveground and belowground biomass of both plant species by an average of 14 and 8%, respectively (above: $F_{1,110}$=24.71, P<0.001, below: $F_{1,110}$=4.22, P=0.0423). Inoculation with AMF tended to increase plant aboveground biomass by 5% ($F_{2,110}$=3.04, P=0.0518), but did not affect belowground biomass (Table 4.1). Overall, *A. incarnata* plants had greater belowground biomasses, but equivalent aboveground biomasses to *A. curassavica* (Table 4.1).

Discussion

We found that AMF have strong, plant-species specific effects on constitutive and aphid-induced VOC emissions. Specifically, we found that 1) high AMF availability increases emissions of total VOCs, GLVs, and methyl salicylate in *A. curassavica* but decreases emissions in *A. incarnata*. 2) Aphids consistently increase emissions of 6-methyl-5-hepten-2-one and benzeneacetaldehyde in both *A. incarnata* and *A. curassavica*, but AMF does not affect these emissions. 3) Aphids suppress emissions of individual terpenes (cis-ocimene, copaene, beta-cubebene, sesquiterpene 2, delta-cadinene) in the absence of AMF. However, high AMF availability suppresses terpene emissions equivalent to aphid-mediated levels, such that aphid damage on plants under high AMF availability does not further suppress terpene emissions. 4) Aphid feeding suppresses cardenolide concentrations only in *A. curassavica,* and AMF do not affect cardenolides in either plant species, despite having strong effects on VOC emissions. Our findings suggest that by altering milkweed VOC profiles, AMF may generate subsequent effects on herbivore and natural enemy attraction, and that AMF may affect the indirect defenses of these milkweed species differently.

Our finding of species-specific effects of AMF on milkweed VOC emissions is consistent with previous studies. For example, AMF increase indirect defenses in one *Medicago trunculata* cultivar, yet suppress indirect defenses in another (Leitner et al. 2010). Similarly, AMF can increase (Fontana et al. 2009) or decrease (Babikova et al. 2014a) GLV emissions, depending on

the plant species. These plant species-specific effects of AMF on VOC production may result from the varying ability of plant species to control carbon allocation to AMF (Grman 2012). In addition, VOC emissions are mediated by interactions among phytohormones (Arimura et al. 2009, Turlings and Erb 2018) that AMF also affect (Jung et al. 2012, Cameron et al. 2013, Gutjahr 2014, Bucher et al. 2014). As milkweed species vary in their hormonal responses in leaves to herbivore damage (Agrawal et al. 2014, Ali and Agrawal 2014), it is possible that the milkweed species may also vary in their responses to AMF inoculation, leading to the observed differences in VOC emissions.

We show for the first time that it is not only the presence of AMF, but the amount AMF inoculum available to plants, that affects VOC emissions. Overall, when compared with zero AMF treatments, medium AMF availability had much weaker effects on VOC emissions than did high AMF availability in both milkweed species. Levels of AMF colonization of plant roots were quite low under medium AMF availability, so there may have been limited nutrient transfer and interactions with phytohormones under medium AMF availability. In the field, milkweed plants have colonization levels ranging from 10 to 80% (Vannette 2011). In this study, plants under medium AMF availability only had, on average, 4% root colonization. Under high AMF availability there was likely greater nutrient exchange between plants and AMF and more substantial interactions with phytohormones (Vannette and Hunter 2011), leading to the stronger effects on VOC emissions. Future work should consider how a broader range of AMF inoculum available to plants affects VOC emissions, as well as herbivore and natural enemy attraction, to evaluate whether there is an optimal level of AMF that promotes plant indirect defenses.

By strongly increasing emissions of total VOCs, GLVs, and methyl salicylate in *A. curassavica*, but decreasing them in *A. incarnata*, AMF may alter herbivore and carnivore attraction to these plant species differently. Particular ratios of constitutive GLVs alter the ability of herbivores to locate their hosts (Visser and Avé 1978, Natale et al. 2003, Bruce et al. 2005), and plants that emit GLVs sustain higher levels of herbivory than do transgenic plants deficient in GLVs (Halitschke et al. 2008). Similarly, high concentrations of methyl salicylate deter some aphid species from colonizing their host plants (Pettersson et al. 1987, Hardie et al. 1994, Babikova et al. 2014a), although others are attracted (Pope et al. 2007). Therefore, AMF-

mediated increases and decreases in constitutive GLV and methyl salicylate emissions likely influence herbivore attraction. In addition, aphid predators and parasitoids are attracted to increased GLV emissions (Whitman and Eller 1990, Du et al. 1998, Wei et al. 2007), and transgenic plants deficient in GLVs experience greater herbivore loads and reduced predator pressure (Halitschke et al. 2008, Schuman et al. 2015). Similarly, methyl salicylate attracts a breadth of aphid predators, including syrphids, lacewings, coccinelids, parasitoid wasps, braconid wasps, and spiders (James 2005, Zhu and Park 2005, Pareja et al. 2009, Rodriguez-Saona et al. 2011, Mallinger et al. 2011), indicating that AMF may affect natural enemy attraction in a plant-species specific manner.

In contrast to the plant species-specific effects of AMF on GLV and methyl salicylate emissions, AMF consistently decreased cis-ocimene emissions in both milkweed species, and also decreased specific sesquiterpenes that were produced only in *A. incarnata*. Our findings of AMF-mediated suppression of specific terpenes confirms similar effects of AMF on terpenoid emissions in *Plantago lanceolata* (Fontana et al. 2009), *Vicia faba* (Babikova et al. 2014b, 2014a), and *Medicago trunculata* (Leitner et al. 2010). Interestingly, AMF-mediated declines in emissions of terpenoid compounds mirror aphid-induced declines in the same compounds in the absence of AMF (Figs. 4.4, 4.5). Particular blends of sesquiterpenes act as cues for natural enemies, as herbivores often induce specific terpenoid blends (Bruce and Pickett 2011, Turlings and Erb 2018). Because plants under high AMF availability exhibited the same profile of terpenoids in the absence of aphids as zero AMF plants with aphids, AMF may improve natural enemy attraction. Indeed mycorrhizal tomato plants without aphids are equally attractive to parasitoids as non-mycorrhizal plants attacked by aphids (Guerrieri et al. 2004), leading to the suggestion that AMF may lead plants to emit profiles indicating herbivore attack in the absence of herbivores (Rasmann et al. 2017).

In contrast to the species-specific effects of AMF on VOC emissions, the effects of aphids on VOC emissions were typically consistent between milkweed species. Our findings that aphids cause only limited induction of HIPVs, and actually suppress emissions of particular VOCs, is consistent with plant responses to aphids in other systems (Staudt et al. 2010, Schwartzberg et al. 2011, Rowen and Kaplan 2016). This lack of induction, and even

suppression of VOCs, may be mediated by aphids not eliciting JA-mediated defenses, or by SA inducing a reduced suite of HIPVs (Walling 2008); oleander aphids alter both SA and JA concentrations in milkweed species (Ali and Agrawal 2014). Despite aphids inducing minor differences in VOC profiles, the differences are likely ecologically relevant. 6-methyl-5-hepten-2-one emissions, which aphids increased strongly (Fig. 4.3), is a known attractant of aphid parasitoids (Du et al. 1998). In addition, 6-methyl-5-hepen-2-one deters aphids, as it indicates low quality plants with high aphid densities (Quiroz et al. 1997). Similarly, evidence suggests that benzeneacetaldehyde, which aphid feeding increased substantially, attracts aphid midge flies (Watanabe et al. 2016), important predators of *A. nerii* in the field (Mohl et al. 2016). Furthermore, although AMF did not alter aphid induction of specific VOCs, by altering GLV and methyl salicylate emissions, AMF altered the overall blend of HIPVs produced. Individual compounds are important in herbivore and natural enemy attraction, but it is more often the particular blend of compounds that matters (Bruce et al. 2005, Bruce and Pickett 2011). For instance, AMF suppress spider mite-induced emissions of methyl salicylate in *Phaseolus vulgaris*, but increase emissions of beta-ocimene and beta-caryophyllene, ultimately leading to increased predatory mite attraction (Schausberger et al. 2012). Future work must consider how AMF mediation of constitutive and aphid-induced volatiles influences herbivore and natural enemy attraction in the field to understand the implications of AMF mediation of milkweed VOC profiles.

Surprisingly, AMF had no effect on the foliar cardenolide concentrations of either milkweed species, despite having strong effects on plant volatile emissions. Our findings confirm those of Fontana et al. (2009), who similarly found that AMF altered constitutive and induced VOC emissions of *Plantago lanceolata*, but did not affect concentrations of direct defenses, iridoid glycosides. However, our findings contrast with previous studies that have found strong effects of AMF availability on *A. curassavica* cardenolide concentrations (Vannette et al. 2013, Tao et al. 2016, Meier and Hunter 2018a, 2018b). We believe that this variation is due to differences in plant age among studies. The plants in this experiment were much younger than those in previous studies, and we have found that AMF do not affect milkweed cardenolide concentrations in 6-week old plants, but do affect cardenolides of 3-month old plants (A. R. Meier, unpublished data). Nonetheless, it is striking that AMF have strong effects on the indirect

defenses of young milkweed plants, but not on direct defenses. Future work should consider how AMF influence allocation to direct and indirect defenses over plant ontogeny.

In conclusion, we found that AMF strongly affect plant constitutive and aphid-induced VOC emissions in a plant-species specific manner, but do not affect plant direct defenses. Our findings suggest that AMF availability may have substantial effects on multitrophic interactions in the field by altering milkweed indirect defenses. However, future work is needed to evaluate whether the AMF-mediated blend of constitutive and aphid-induced volatiles alters herbivore and natural enemy attraction in the field.

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Table 4.1. Effects of plant species, the availability of arbuscular mycorrhizal fungi (AMF) inoculum, aphid feeding, and their interactions on plant traits, including the proportion of roots colonized by any AMF structures and by arbuscules only, natural log-transformed foliar cardenolide concentration (mg/g), cardenolide diversity, cardenolide polarity, aboveground biomass (mg), and natural log-transformed belowground biomass (mg). Numbers represent F-values and P-values from linear mixed models. Final sample sizes per treatment were 12 plants. **P < 0.001, *P < 0.05, †P < 0.1.

Plant Trait	Plant species	AMF	Aphids	Plant species x AMF	AMF x Aphid	Plant species x Aphid	Plant species x AMF x Aphid
AMF colonization							
Proportion root colonization	$F_{1,22}$=0.54 P=0.4719	$F_{2,110}$=23.5 P<.001**	$F_{1,110}$=1.51 0.2211	$F_{2,110}$=0.27 P=0.7653	$F_{2,110}$=0.66 P=0.5182	$F_{1,110}$=1.35 P=0.2471	$F_{2,110}$=1.48 P=0.2313
Proportion arbuscules	$F_{1,22}$=2.67 P=0.1164	$F_{2,110}$=12.42 P<.001**	$F_{1,110}$=1.85 0.1765	$F_{2,110}$=0.8 P=0.4512	$F_{2,110}$=0.68 P=0.5104	$F_{1,110}$=1.55 P=0.2158	$F_{2,110}$=1.2 P=0.3037
Cardenolides							
Cardenolide concentration	$F_{1,22}$=616.08 P<.001**	$F_{2,110}$=0.73 P=0.4855	$F_{1,110}$=3.61 0.0601†	$F_{2,110}$=0.92 P=0.4006	$F_{2,110}$=1.1 P=0.3375	$F_{1,110}$=6.95 P=0.0096**	$F_{2,110}$=0.31 P=0.7339
Cardenolide diversity	$F_{1,21.2}$=2514.66 P<.001***	$F_{2,99.6}$=0.11 P=0.8939	$F_{1,98.8}$=0.19 0.6618	$F_{2,99.6}$=0.49 P=0.6165	$F_{2,99.4}$=0 P=0.9961	$F_{1,98.8}$=0.59 P=0.444	$F_{2,99.4}$=0.57 P=0.5665
Cardenolide polarity	$F_{1,21.8}$=6.49 P=0.0185*	$F_{2,98.6}$=2.32 P=0.1039	$F_{1,98.3}$=2.8 P=0.0973†	$F_{2,98.6}$=1.09 P=0.3402	$F_{2,98.6}$=0.38 P=0.6817	$F_{1,98.3}$=0.09 P=0.7667	$F_{2,98.6}$=0.06 P=0.9399
Biomass							
Aboveground biomass	$F_{1,22}$=0.36 0.5539	$F_{2,110}$=3.04 P=0.0518†	$F_{1,110}$=24.71, P<.001**	$F_{2,110}$=1.71 0.185	$F_{2,110}$=0.24 P=0.7898	$F_{1,110}$=0.1 P=0.7528	$F_{2,110}$=0.39 P=0.6808
Belowground biomass	$F_{1,22}$=43.63 P<.001**	$F_{2,110}$=0.51 P=0.6035	$F_{1,110}$=4.22 P=0.0423*	$F_{2,110}$=0.11 P=0.8984	$F_{2,110}$=1.09 P=0.3382	$F_{1,110}$=0.05 P=0.8249	$F_{2,110}$=2.4 P=0.0957†

Table 4.2. Effects of plant species, the availability of arbuscular mycorrhizal fungi (AMF) inoculum, aphid feeding, and their interactions on VOC emissions. A "C" in the Species ("Spp") column indicates that the compound was found in *A. curassavica*, and "I" indicates that the compound was found in *A. incarnata*. Numbers represent F-values and P-values from linear mixed models. Final sample sizes were 12 plants per treatment. **P < 0.001, *P < 0.05, †P < 0.1.

Dependent Variable	Spp	Plant spp	AMF	Aphids	PCA1	PCA2	Plant spp x AMF	AMF x Aphids	Plant spp x Aphids	Plant spp x AMF x Aphids
Total VOC Concentration	C,I	$F_{1,19.9}$=7.32 P=0.0136*	$F_{2,107.7}$=0.04 0.9627	$F_{1,108.1}$=0.86 P=0.3562	$F_{1,19.9}$=0.71 P=0.4108	$F_{1,19.9}$=0.04 P=0.8381	$F_{2,108.2}$=3.48 P=0.0343*	$F_{2,106.8}$=0.41 P=0.6672	$F_{1,108.9}$=0.59 P=0.4443	$F_{2,108.3}$=1.15 P=0.3207
Ketone	C,I	$F_{1,20}$=2.96 P=0.1009	$F_{2,107}$=0.5 P=0.6065	$F_{1,107.5}$=9.74 P=0.0023**	$F_{1,20}$=5.63 P=0.0278*	$F_{1,20}$=0.19 P=0.6678	$F_{2,107.6}$=0.38 P=0.688	$F_{2,106}$=0.17 P=0.8436	$F_{1,108.4}$=1.43 P=0.2352	$F_{2,107.7}$=1.95 P=0.1474
6-methyl-5-hepten-2-one	C,I	$F_{1,20}$=0.15 P=0.7042	$F_{2,110}$=1.37 P=0.2583	$F_{1,110}$=6.15 P=0.0147*	$F_{1,20}$=2.01 P=0.1716	$F_{1,20}$=1.52 P=0.2319	$F_{2,110}$=0.04 P=0.9628	$F_{2,110}$=0 P=0.9951	$F_{1,110}$=0.26 P=0.6084	$F_{2,110}$=0.41 P=0.6637
6,10,14-Trimethyl pentadecan-2-one	C,I	$F_{1,20}$=86.13 P=<.001**	$F_{2,107}$=1.15 P=0.3191	$F_{1,107.5}$=1.1 P=0.2962	$F_{1,20.1}$=17.1 P=0.0005**	$F_{1,20}$=18.57 P=0.0003**	$F_{2,107.6}$=2.84 P=0.063†	$F_{2,106.2}$=0.35 P=0.7084	$F_{1,108.2}$=3.22 P=0.0755†	$F_{2,107.6}$=1.67 P=0.1927
Monoterpenoids	C,I	$F_{1,20}$=7.5 P=0.0127**	$F_{2,105.2}$=1.02 P=0.3645	$F_{1,105.4}$=0.39 P=0.5321	$F_{1,20}$=2.78 P=0.1112	$F_{1,20}$=0 P=0.9582	$F_{2,105.4}$=0.16 P=0.8494	$F_{2,104.9}$=1.45 P=0.2399	$F_{1,105.7}$=0.04 P=0.8403	$F_{2,105.4}$=0.08 P=0.9241
3-Carene	C,I	$F_{1,19.8}$=0.04 P=0.8465	$F_{2,104.6}$=0.38 P=0.6834	$F_{1,104.6}$=1.54 P=0.2172	$F_{1,19.9}$=6.79 P=0.0169*	$F_{1,19.8}$=4.92 P=0.0384*	$F_{2,104.7}$=0.15 P=0.8644	$F_{2,104.5}$=0.86 P=0.4253	$F_{1,104.7}$=0 P=0.9984	$F_{2,104.6}$=0.94 P=0.3954
cis-Ocimene	C,I	$F_{1,20}$=8.07 P=0.0101*	$F_{2,110}$=2.57 P=0.0811†	$F_{1,110}$=4.97 P=0.0279*	$F_{1,20}$=0.02 P=0.8986	$F_{1,20}$=1.2 P=0.2871	$F_{2,110}$=1.06 P=0.3491	$F_{2,110}$=3.14 P=0.0472*	$F_{1,110}$=0.06 P=0.8063	$F_{2,110}$=0.09 P=0.9115
Green Leafy Volatiles	C,I	$F_{1,20}$=10.74 P=0.0038**	$F_{2,108.3}$=0.12 P=0.886	$F_{1,108.9}$=0.01 P=0.943	$F_{1,20}$=0.58 P=0.4536	$F_{1,20}$=0.01 P=0.9399	$F_{2,109}$=3.5 P=0.0336*	$F_{2,107.2}$=0.3 P=0.7444	$F_{1,109.6}$=0.43 P=0.5157	$F_{2,109.1}$=0.75 P=0.4748
3-hexenyl-acetate	C,I	$F_{1,20}$=12.55 P=0.0021**	$F_{2,108.3}$=0.1 P=0.9052	$F_{1,108.9}$=0.03 P=0.8641	$F_{1,20}$=0.53 P=0.4765	$F_{1,20}$=0 P=0.978	$F_{2,109}$=3.6 P=0.0307*	$F_{2,107.2}$=0.29 P=0.7473	$F_{1,109.6}$=0.23 P=0.634	$F_{2,109.1}$=0.81 P=0.4458
Hexyl acetate	C,I	$F_{1,20}$=0.61 P=0.4422	$F_{2,108}$=0.44 P=0.6475	$F_{1,108.5}$=0 P=0.9922	$F_{1,20}$=0.02 P=0.8978	$F_{1,20}$=0.16 P=0.6901	$F_{2,108.6}$=3.29 P=0.0411*	$F_{2,106.9}$=0.4 P=0.6695	$F_{1,109.3}$=1.95 P=0.1654	$F_{2,108.7}$=0.72 P=0.4877
(2Z)-2-Hexenyl acetate	C,I	$F_{1,20}$=15.58 P=0.0008**	$F_{2,110}$=0.29 P=0.7513	$F_{1,110}$=0.88 P=0.3509	$F_{1,20}$=0.5 P=0.4881	$F_{1,20}$=0.05 P=0.833	$F_{2,110}$=2.44 P=0.0918†	$F_{2,110}$=0.27 P=0.764	$F_{1,110}$=0.93 P=0.3373	$F_{2,110}$=0.59 P=0.5556
Benzyl alcohol	I		$F_{2,51}$=2.63 P=0.0821†	$F_{1,53.9}$=0.05 P=0.8277	$F_{1,9}$=0.18 P=0.6854	$F_{1,9}$=0.67 P=0.4332		$F_{2,50.9}$=0.7 P=0.4996		
Unknown GLV	C,I	$F_{1,20}$=0.25 P=0.6246	$F_{2,108.3}$=0.7 P=0.5002	$F_{1,108.8}$=1.77 P=0.1867	$F_{1,20}$=2.84 P=0.1076	$F_{1,20}$=0.91 P=0.3528	$F_{2,108.9}$=0.75 P=0.4729	$F_{2,107.1}$=1.04 P=0.3577	$F_{1,109.6}$=1.08 P=0.3012	$F_{2,109.1}$=2.41 P=0.0944†
Benzenoids/ Phenylpropanoid	C,I	$F_{1,19.9}$=12.52 P=0.0021**	$F_{2,107}$=0.05 P=0.9477	$F_{1,107.4}$=0.41 P=0.5229	$F_{1,20}$=0.8 P=0.3828	$F_{1,20}$=0.15 P=0.7021	$F_{2,107.5}$=3.19 P=0.0449*	$F_{2,106.3}$=0.64 P=0.5289	$F_{1,108.1}$=0.29 P=0.5945	$F_{2,107.6}$=0.81 P=0.4485
Benzeneacetalde-hyde	C,I	$F_{1,19.9}$=2.16 P=0.1577	$F_{2,106.8}$=0.73 P=0.483	$F_{1,107.1}$=116.1 P=<.001**	$F_{1,19.9}$=4.02 P=0.0586†	$F_{1,19.9}$=2.62 P=0.1215	$F_{2,107.2}$=1.94 P=0.1485	$F_{2,106.2}$=0.48 P=0.6228	$F_{1,107.7}$=3.48 P=0.0648	$F_{2,107.2}$=0.54 P=0.584

Table 4.2, continued

Methyl ester benzoic acid	C,I	$F_{1,20}$=24.15 P=<.0001**	$F_{2,107.6}$=0.45 P=0.6357	$F_{1,108.1}$=1.95 P=0.1653	$F_{1,20}$=0.42 P=0.526	$F_{1,20}$=0.38 P=0.5438	$F_{2,108.2}$=0.53 P=0.5881	$F_{2,106.6}$=1.31 P=0.2737	$F_{1,108.9}$=0.09 P=0.7662	$F_{2,108.3}$=1.7 P=0.1881
Phenylmethyl ester acetic acid	I		$F_{2,55}$=1.98 P=0.1477	$F_{1,55}$=0.21 P=0.6491	$F_{1,9}$=0.71 P=0.4209	$F_{1,9}$=0.27 P=0.6181		$F_{2,55}$=0.64 P=0.5293		
Methyl salicylate	C,I	$F_{1,19.9}$=10.67 P=0.0039**	$F_{2,107.1}$=0.06 P=0.9378	$F_{1,107.5}$=0.53 P=0.4687	$F_{1,20}$=0.64 P=0.4335	$F_{1,19.9}$=0.16 P=0.6917	$F_{2,107.6}$=3.31 P=0.0402*	$F_{2,106.3}$=0.5 P=0.6084	$F_{1,108.2}$=0.29 P=0.5883	$F_{2,107.6}$=0.64 P=0.53
Methyl 2-(methoxy methyl)benzoate	I		$F_{2,51.5}$=1.52 P=0.2289	$F_{1,54}$=0.23 P=0.6371	$F_{1,9}$=0.09 P=0.7663	$F_{1,8.9}$=0.05 P=0.8301		$F_{2,51.4}$=1 P=0.3746		
Benzyl butyrate	I		$F_{2,51.6}$=1.38 P=0.261	$F_{1,54.1}$=2.57 P=0.1145	$F_{1,9}$=0.07 P=0.7957	$F_{1,9}$=0.47 P=0.5107		$F_{2,51.5}$=0.94 P=0.3971		
3-hexen-1-ol-benzoate	C,I	$F_{1,19.9}$=24.86 P<.001**	$F_{2,106.4}$=0.64 P=0.5309	$F_{1,106.7}$=0.01 P=0.9328	$F_{1,20}$=1.35 P=0.2595	$F_{1,19.9}$=0 P=0.9618	$F_{2,106.8}$=0.8 P=0.4503	$F_{2,105.8}$=1.29 P=0.2789	$F_{1,107.3}$=0.19 P=0.6642	$F_{2,106.8}$=0.77 P=0.4669
trans-2-hexenyl benzoate	I		$F_{2,51.6}$=0.85 P=0.4318	$F_{1,54.4}$=5.01 P=0.0293*	$F_{1,9}$=1.07 P=0.3289	$F_{1,9}$=0.28 P=0.6097		$F_{2,51.5}$=1.29 P=0.2846		
cis-3-Hexenyl salicylate	I		$F_{2,51}$=0.47 P=0.6276	$F_{1,53.4}$=1 P=0.3227	$F_{1,9}$=1.28 P=0.2872	$F_{1,9}$=0 P=0.9876		$F_{2,50.9}$=1.22 P=0.3028		
Benzyl benzoate	C,I	$F_{1,19.9}$=17.61 P=0.0004**	$F_{2,106.5}$=0.11 P=0.8921	$F_{1,106.8}$=0 P=0.9881	$F_{1,20}$=0.62 P=0.442	$F_{1,19.9}$=0.1 P=0.7548	$F_{2,106.9}$=1.18 P=0.3127	$F_{2,105.9}$=1.45 P=0.2387	$F_{1,107.4}$=0.01 P=0.9098	$F_{2,106.9}$=2.11 P=0.1263
Sesquiterpenoids	C,I	$F_{1,20}$=32.64 P<.0001**	$F_{2,108.3}$=0.34 P=0.7138	$F_{1,108.8}$=0.15 P=0.6952	$F_{1,20}$=1 P=0.3288	$F_{1,20}$=0.15 P=0.6985	$F_{2,108.9}$=2.58 P=0.08†	$F_{2,107.1}$=1.5 P=0.2286	$F_{1,109.6}$=2.09 P=0.1514	$F_{2,109}$=1.3 P=0.2757
Copaene	I		$F_{2,55}$=0.14 P=0.8716	$F_{1,55}$=6.28 P=0.0152*	$F_{1,9}$=0.44 P=0.5255	$F_{1,9}$=0.83 P=0.3867		$F_{2,55}$=5.88 P=0.0048**		
beta-Bourbonene	I		$F_{2,52.1}$=2.2 P=0.1206	$F_{1,54.9}$=0.85 P=0.3605	$F_{1,9}$=0.1 P=0.7627	$F_{1,9}$=0.54 P=0.483		$F_{2,52}$=0.06 P=0.9402		
beta-Cubebene	I		$F_{2,52.6}$=0.01 P=0.9883	$F_{1,55}$=3.93 P=0.0524†	$F_{1,9}$=0 P=0.9984	$F_{1,9}$=1.7 P=0.2252		$F_{2,52.4}$=3.46 P=0.0388*		
Caryophyllene	I		$F_{2,55}$=1.66 P=0.1997	$F_{1,55}$=2.63 P=0.1109	$F_{1,9}$=0.02 P=0.8877	$F_{1,9}$=0.04 P=0.8432		$F_{2,55}$=2.61 P=0.0825†		
alpha-Bergamotene	I		$F_{2,50.9}$=2.06 P=0.1378	$F_{1,53.3}$=0.57 P=0.4524	$F_{1,9}$=2.51 P=0.1473	$F_{1,9}$=1.03 P=0.3367		$F_{2,50.8}$=0.75 P=0.4783		
Sesquiterpene 1	I		$F_{2,52.2}$=1.34 P=0.2703	$F_{1,54.9}$=0.52 P=0.4723	$F_{1,9}$=2.58 P=0.1431	$F_{1,9}$=0.28 P=0.6117		$F_{2,52}$=0.68 P=0.5092		
beta-Farnesene	I		$F_{2,51.4}$=1.08 P=0.3463	$F_{1,53.9}$=0.19 P=0.6671	$F_{1,9}$=0.28 P=0.6126	$F_{1,9}$=0.19 P=0.6713		$F_{2,51.4}$=1 P=0.3746		
Germacrene D	I		$F_{2,52.7}$=1.8 P=0.176	$F_{1,55}$=3.35 P=0.0724†	$F_{1,9}$=0.16 P=0.6997	$F_{1,9}$=2.33 P=0.1615		$F_{2,52.5}$=1.17 P=0.3192		
(Z,E)-alpha-Farnesene	I		$F_{2,55}$=0.34 P=0.7107	$F_{1,55}$=0.97 P=0.3301	$F_{1,9}$=0.69 P=0.4276	$F_{1,9}$=0.82 P=0.3899		$F_{2,55}$=0.6 P=0.5546		
alpha-Farnesene	I		$F_{2,55}$=1.14 P=0.3272	$F_{1,55}$=0.25 P=0.6183	$F_{1,9}$=0.36 P=0.5607	$F_{1,9}$=0.64 P=0.4448		$F_{2,55}$=0.72 P=0.4931		
Sesquiterpene 2	I		$F_{2,51.5}$=0.79 P=0.4602	$F_{1,54.6}$=5.22 P=0.0262**	$F_{1,9}$=0.42 P=0.5314	$F_{1,9}$=1.33 P=0.2782		$F_{2,51.4}$=8.71 P=0.0006**		

Table 4.2, continued

delta-Cadinene	I		$F_{2,55}$=0.17 P=0.846	$F_{1,55}$=8.81 P=0.0044***	$F_{1,9}$=0.01 P=0.9185	$F_{1,9}$=2.41 P=0.1548		$F_{2,55}$=5.47 P=0.0068**		
Sesquiterpene 3	I		$F_{2,55}$=1.84 P=0.1684	$F_{1,55}$=0.49 P=0.4872	$F_{1,9}$=3.29 P=0.1029	$F_{1,9}$=1.37 P=0.2718		$F_{2,55}$=1.63 P=0.2047		
Sesquiterpene 4	C,I	$F_{1,20}$=3.47 P=0.0774†	$F_{2,107.2}$=1.2 P=0.3051	$F_{1,107.7}$=1.78 P=0.185	$F_{1,20}$=0.64 P=0.4345	$F_{1,20}$=0.23 P=0.6341	$F_{2,107.9}$=2.41 P=0.0945†	$F_{2,106.1}$=1.09 P=0.3404	$F_{1,108.6}$=1.17 P=0.281	$F_{2,107.9}$=0.1 P=0.9052
(+/-)-trans-Nerolidol	I		$F_{2,52.2}$=0.62 P=0.5444	$F_{1,54.8}$=1.06 P=0.3069	$F_{1,9}$=0.18 P=0.6839	$F_{1,9}$=0.3 P=0.5974		$F_{2,52.1}$=1.03 P=0.3639		
trans,trans-Farnesal	C		$F_{2,53.3}$=2.83 P=0.0676†	$F_{1,51.2}$=2.59 P=0.1138	$F_{1,9}$=2.72 P=0.1333	$F_{1,9}$=1.51 P=0.2497		$F_{2,52.8}$=0.2 P=0.8182		
Other	I									
Isophytol (alcohol)	I		$F_{2,51.2}$=0.3 P=0.7451	$F_{1,53.7}$=0.14 P=0.7114	$F_{1,9}$=0.02 P=0.8981	$F_{1,9}$=0.63 P=0.4476		$F_{2,51.2}$=1.7 P=0.1936		
Unknowns	C,I									
Unknown 1	I		$F_{2,51.7}$=0.43 P=0.65	$F_{1,54.5}$=1.66 P=0.2027	$F_{1,9}$=1.12 P=0.3175	$F_{1,9}$=0.06 P=0.8187		$F_{2,51.6}$=1.38 P=0.2599		
Unknown 2	C,I	$F_{1,20}$=3.87 P=0.0633†	$F_{2,110}$=0.01 P=0.9933	$F_{1,110}$=0.1 P=0.7567	$F_{1,20}$=1.52 P=0.2326	$F_{1,20}$=0.12 P=0.7379	$F_{2,110}$=1.71 P=0.1859	$F_{2,110}$=0.94 P=0.3937	$F_{1,110}$=1.89 P=0.1717	$F_{2,110}$=0.53 P=0.5927
Unknown 3	I		$F_{2,51.1}$=1.74 P=0.1855	$F_{1,54.1}$=2.18 P=0.1455	$F_{1,9}$=0.01 P=0.9255	$F_{1,9}$=1.22 P=0.2986		$F_{2,51}$1.01 P=0.3697		
Unknown 4	I		$F_{2,51.6}$=1.56 P=0.219	$F_{1,55}$=0.03 P=0.8542	$F_{1,9}$=1.94 P=0.1976	$F_{1,9}$=0.01 P=0.9293		$F_{2,51.4}$=0.98 P=0.3826		
Unknown 5	I		$F_{2,51.2}$=0.32 P=0.7286	$F_{1,53.8}$=1.26 P=0.2674	$F_{1,9}$=0.31 P=0.5914	$F_{1,9}$=0.28 P=0.612		$F_{2,51.1}$=1.46 P=0.2407		
Unknown 6	I		$F_{2,51.8}$=0.72 P=0.4906	$F_{1,54.3}$=0.93 P=0.3398	$F_{1,9}$=0.48 P=0.5055	$F_{1,9}$=0.36 P=0.5627		$F_{2,51.7}$=1.69 P=0.1948		
Unknown 7	I		$F_{2,55}$=0.16 P=0.8517	$F_{1,55}$=1.02 P=0.3174	$F_{1,9}$=0.52 P=0.4907	$F_{1,9}$=0.08 P=0.7779		$F_{2,55}$=0.72 P=0.4919		
Unknown 8	I		$F_{2,52.3}$=0.71 P=0.4977	$F_{1,54.9}$=0.77 P=0.385	$F_{1,9}$=0.15 P=0.7097	$F_{1,9}$=0.3 P=0.5981		$F_{2,52.1}$=0.78 P=0.464		
Unknown 9	C,I	$F_{1,20}$=15.46 P=0.0008**	$F_{2,107.2}$=0.12 P=0.8869	$F_{1,107.6}$=0.27 P=0.6036	$F_{1,20}$=1.02 P=0.3249	$F_{1,19.9}$=0.66 P=0.4259	$F_{2,107.7}$=4.56 P=0.0126*	$F_{2,106.5}$=1.11 P=0.3335	$F_{1,108.4}$=2.66 P=0.1059	$F_{2,107.8}$=1.69 P=0.1891
Unknown 10	I		$F_{2,51.4}$=1.22 P=0.3037	$F_{1,54.6}$=2.45 P=0.1231	$F_{1,9}$=2.29 P=0.1643	$F_{1,9}$=0.01 P=0.9218		$F_{2,51.3}$=4.84 P=0.0118*		
Unknown 11	I		$F_{2,52}$=0.5 P=0.6094	$F_{1,54.7}$=0.64 P=0.426	$F_{1,9}$=0.58 P=0.466	$F_{1,9}$=0.06 P=0.8192		$F_{2,51.9}$=1.57 P=0.2186		
Unknown 12	I		$F_{2,51.2}$=0.3 P=0.7451	$F_{1,53.7}$=0.14 P=0.7114	$F_{1,9}$=0.02 P=0.8981	$F_{1,9}$=0.63 P=0.4476		$F_{2,51.2}$=1.7 P=0.1936		
Unknown 13	I		$F_{2,52.9}$=1.53 P=0.2256	$F_{1,55}$=0.35 P=0.5539	$F_{1,9}$=0.07 P=0.7959	$F_{1,9}$=0.23 P=0.6445		$F_{2,52.7}$=2.51 P=0.091†		

Table 4.3. The mean amounts (ng gDW^{-1} h^{-1}) and standard error of VOCs collected from two milkweed species in the presence and absence of aphids, under zero, medium, and high levels of AMF inoculum availability. A "S" in the Standard ("Stnd") column indicates that compound identity was verified with a standard. A "C" in the Species ("Spp") column indicates that the compound was found in *A. curassavica*, and "I" indicates that the compound was found in *A. incarnata*. LRI indicates the linear retention index calculated for each compound.

				A. curassavica						***A. incarnata***					
				Zero		**Medium**		**High**		**Zero**		**Medium**		**High**	
	Stnd	**Spp**	**LRI**	**None**	**Aphids**	**None**	**Aphids**	**None**	**Aphids**	**None**	**Aphids**	**None**	**Aphids**	**None**	**Aphids**
Total VOC Concentration		C,I		4.7148± 1.2809	4.4255± 1.4103	3.5247± 0.9757	5.0154± 0.9223	5.8097± 1.3157	8.4446± 2.4769	27.5671± 8.0676	19.234± 6.2451	27.7414± 8.7337	19.7584± 5.5464	13.6063± 5.3702	20.6515± 6.3036
Ketone		C,I		0.0902± 0.023	0.1189± 0.029	0.0612± 0.0191	0.1175± 0.026	0.0755± 0.0226	0.1266± 0.0365	0.1675± 0.0461	0.222± 0.0603	0.1765± 0.037	0.1677± 0.0388	0.1331± 0.0395	0.1851± 0.039
6-methyl-5-hepten-2-one	S	C,I	987	0.0762± 0.022	0.1043± 0.0275	0.0486± 0.0184	0.0987± 0.0263	0.0619± 0.0226	0.1023± 0.0343	0.1128± 0.0436	0.1664± 0.0527	0.1015± 0.0292	0.1107± 0.0295	0.0893± 0.0367	0.1361± 0.038
6,10,14-Trimethylpentadecan-2-one		C,I	1843	0.0141± 0.0029	0.0146± 0.0029	0.0126± 0.0023	0.0188± 0.0024	0.0136± 0.0011	0.0243± 0.0061	0.0547± 0.0104	0.0556± 0.01	0.075± 0.0198	0.057± 0.0123	0.0439± 0.0051	0.049± 0.0084
Monoterpenoids		C,I		0.0329± 0.0096	0.1103± 0.0963	0.0249± 0.0176	0.0312± 0.0145	0.0261± 0.0099	0.0498± 0.034	0.1411± 0.0436	0.0786± 0.031	0.0942± 0.0276	0.0796± 0.0294	0.0471± 0.0126	0.095± 0.0397
3-Carene	S	C,I	1006	0.0221± 0.0094	0.1073± 0.0958	0.019±0 .0171	0.0264± 0.0138	0.0186± 0.0083	0.0456± 0.0337	0.0121± 0.0057	0.0397± 0.0273	0.033± 0.0215	0.0091± 0.004	0.0133± 0.0049	0.0324± 0.0172
cis-Ocimene	S	C,I	1036	0.0107± 0.0024	0.003± 0.0012	0.0059± 0.0018	0.0048± 0.0015	0.0076± 0.0031	0.0042± 0.0015	0.129± 0.0435	0.0389± 0.013	0.0611± 0.0234	0.0705± 0.0304	0.0338± 0.0137	0.0626± 0.0398
Green Leafy Volatiles		C,I		0.2265± 0.0651	0.2094± 0.067	0.1373± 0.042	0.2034± 0.0531	0.2935± 0.0804	0.4107± 0.1638	1.5059± 0.4897	1.2654± 0.411	2.1897± 0.7918	1.4315± 0.501	0.9763± 0.4022	1.206± 0.4873
3-hexenyl-acetate	S	C,I	1009	0.1732± 0.0563	0.1323± 0.0463	0.0993± 0.0331	0.1414± 0.0392	0.2283± 0.0699	0.3189± 0.128	1.3017± 0.4309	1.0989± 0.3626	1.8732± 0.6712	1.2307± 0.4393	0.8617± 0.3518	1.0379± 0.4291
Hexyl acetate		C,I	1018	0.0135± 0.0033	0.0184± 0.0085	0.0086± 0.0021	0.0144± 0.0034	0.0158± 0.0042	0.0282± 0.0105	0.0434± 0.0158	0.0321± 0.0114	0.0942± 0.0474	0.0514± 0.02	0.0282± 0.0138	0.0352± 0.0159
(2Z)-2-Hexenyl acetate		C,I	1021	0.0019± 0.0008	0.0018± 0.0011	0.001± 0.0007	0.0014± 0.0007	0.003± 0.0014	0.0047± 0.0027	0.0282± 0.0096	0.0218± 0.0081	0.0632± 0.0341	0.0326± 0.0127	0.022± 0.0099	0.0205± 0.0092
Benzyl alcohol	S	I	1033	0±0	0±0	0±0	0±0	0±0	0±0	0.0883± 0.0301	0.0545± 0.0184	0.1059± 0.0427	0.0871± 0.029	0.0371± 0.0197	0.0643± 0.0227
Unknown GLV		C,I	1038	0.0379± 0.0106	0.0568± 0.0193	0.0284± 0.0081	0.0462± 0.0129	0.0464± 0.0121	0.0588± 0.0281	0.0442± 0.0153	0.0583± 0.0161	0.0531± 0.0143	0.0296± 0.0077	0.0274± 0.0091	0.0482± 0.0137
Benzenoids/ phenylpropanoids		C,I		2.702± 0.8189	2.4086± 0.8267	1.9899± 0.6132	2.8462± 0.6263	3.573± 0.8916	5.1045± 1.6702	19.7531± 6.0866	14.2519± 4.7214	19.5689± 6.332	14.154± 3.9174	9.6861± 3.9544	14.8821± 4.5006

Table 4.3, continued

Benzeneacetal-dehyde	S	C,I	1043	0.0004± 0.0001	0.0062± 0.0012	0.0009± 0.0005	0.0054± 0.002	0.0007± 0.0004	0.0051± 0.001	0.0021± 0.0009	0.0056± 0.0012	0.0023± 0.0005	0.0081± 0.0016	0.0011± 0.0003	0.0046± 0.0008
Methyl ester benzoic acid	S	C,I	1089	0.0155± 0.0048	0.0063± 0.0033	0.0126± 0.0047	0.0108± 0.0026	0.0186± 0.0047	0.0194± 0.0072	0.515± 0.2851	0.22± 0.0724	0.5491± 0.2339	0.2503± 0.0819	0.2935± 0.137	0.4576± 0.1792
Phenylmethyl ester acetic acid	S	I	1164	0±0	0±0	0±0	0±0	0±0	0±0	0.2062± 0.0888	0.1369± 0.0609	0.4496± 0.2573	0.1912± 0.0707	0.0942± 0.0529	0.1764± 0.0946
Methyl salicylate	S	C,I	1190	2.5581± 0.7948	2.3463± 0.8149	1.94± 0.6002	2.7522± 0.6146	3.4675± 0.8775	4.9858± 1.6397	15.0276± 4.2483	11.4047± 3.4454	14.6808± 4.3692	11.3846± 3.0775	7.6556± 2.9895	11.3502± 3.3215
Methyl 2-(methoxymet-hyl)benzoate	S	I	1339	0±0	0±0	0±0	0±0	0±0	0±0	0.0276± 0.0079	0.0194± 0.0068	0.0325± 0.0097	0.0415± 0.0162	0.0187± 0.0104	0.0384± 0.0203
Benzyl butyrate	S	I	1345	0±0	0±0	0±0	0±0	0±0	0±0	0.0256± 0.0111	0.0064± 0.0054	0.0656± 0.0464	0.0154± 0.0078	0.015± 0.0093	0.013± 0.0081
3-hexen-1-ol-benzoate	S	C,I	1570	0.0643± 0.0287	0.021±0 .0078	0.0154± 0.0074	0.0399± 0.0148	0.0308± 0.0127	0.0336± 0.011	1.8039± 0.7074	1.115± 0.5634	1.5002± 0.5856	1.0092± 0.3442	0.7857± 0.3896	1.1564± 0.3987
trans-2-hexenyl benzoate		I	1583	0±0	0±0	0±0	0±0	0±0	0±0	0.0339± 0.0081	0.02± 0.0108	0.0468± 0.0306	0.0202± 0.0085	0.0192± 0.0091	0.0137± 0.004
cis-3-Hexenyl salicylate		I	1668	0±0	0±0	0±0	0±0	0±0	0±0	0.0395± 0.0151	0.0216± 0.0131	0.03± 0.0126	0.0253± 0.0142	0.0173± 0.0088	0.0198± 0.008
Benzyl benzoate	S	C,I	1763	0.0637± 0.0269	0.0287± 0.0108	0.021±0 .0111	0.0378± 0.0107	0.0554± 0.0159	0.0604± 0.0214	2.0718± 0.939	1.3021± 0.6347	2.212± 0.9229	1.2083± 0.4124	0.7858± 0.3866	1.652± 0.7274
Sesquiterpen-oids		C,I		0.1001± 0.0264	0.0888± 0.0283	0.0714± 0.0217	0.1054± 0.0202	0.1033± 0.0238	0.1512± 0.0364	3.103± 0.8601	1.6195± 0.5676	3.0011± 0.9229	2.145± 0.744	1.3978± 0.5997	2.3011± 0.8287
Copaene		I	1374	0±0	0±0	0±0	0±0	0±0	0±0	0.2332± 0.0794	0.0354± 0.0205	0.3331± 0.1255	0.0487± 0.0353	0.0935± 0.0633	0.2659± 0.1217
beta-Bourbonene		I	1383	0±0	0±0	0±0	0±0	0±0	0±0	0.0055± 0.0034	0.005± 0.005	0.0197± 0.0122	0.007± 0.0042	0.0038± 0.0029	0.0014± 0.0009
beta-Cubebene		I	1389	0±0	0±0	0±0	0±0	0±0	0±0	0.0292± 0.0092	0.0066± 0.0029	0.0373± 0.0167	0.0157± 0.0086	0.0114± 0.0048	0.0263± 0.0109
Caryophyllene	S	I	1418	0±0	0±0	0±0	0±0	0±0	0±0	0.0817± 0.0292	0.0217± 0.008	0.0777± 0.0357	0.0462± 0.0138	0.026± 0.0072	0.0421± 0.0133
alpha-Bergamotene		I	1430	0±0	0±0	0±0	0±0	0±0	0±0	0.0235± 0.0092	0.0159± 0.007	0.0274± 0.0102	0.0405± 0.0212	0.0086± 0.0037	0.0179± 0.0076
Sesquiterpene 1		I	1439	0±0	0±0	0±0	0±0	0±0	0±0	0.0868± 0.031	0.1173± 0.0484	0.0746± 0.0175	0.0643± 0.0202	0.0613± 0.0315	0.0868± 0.0271
beta-Farnesene		I	1457	0±0	0±0	0±0	0±0	0±0	0±0	0.0336± 0.0106	0.0186± 0.007	0.0366± 0.0136	0.0319± 0.0147	0.0147± 0.0065	0.0224± 0.0092

Table 4.3, continued

Germacrene D		I	1480	0±0	0±0	0±0	0±0	0±0	0±0	0.1291± 0.0897	0.0163± 0.0062	0.1064± 0.0436	0.0866± 0.0565	0.0253± 0.0132	0.034± 0.0171
(Z,E)-alpha-Farnesene	S	I	1494	0±0	0±0	0±0	0±0	0±0	0±0	0.072± 0.0211	0.042± 0.0173	0.0764± 0.0275	0.0471± 0.0169	0.0397± 0.018	0.0525± 0.0195
alpha-Farnesene	S	I	1509	0±0	0±0	0±0	0±0	0±0	0±0	1.9675± 0.5675	1.1519± 0.4155	1.6532± 0.481	1.3929± 0.4348	0.9119± 0.373	1.3473± 0.4735
Sesquiterpene 2		I	1515	0±0	0±0	0±0	0±0	0±0	0±0	0.0212± 0.0078	0.0029± 0.0014	0.0399± 0.0181	0.0065± 0.0041	0.0086± 0.0071	0.023± 0.0092
delta-Cadinene		I	1523	0±0	0±0	0±0	0±0	0±0	0±0	0.1614± 0.054	0.0257± 0.0124	0.2184± 0.0842	0.0399± 0.0265	0.0585± 0.0377	0.1683± 0.0837
Sesquiterpene 3		I	1533	0±0	0±0	0±0	0±0	0±0	0±0	0.0125± 0.0068	0.0046± 0.0046	0.0134± 0.008	0.0074± 0.005	0±0	0.0031± 0.0024
Sesquiterpene 4		C,I	1560	0.0664± 0.0198	0.0552± 0.0205	0.0466± 0.016	0.0664± 0.0137	0.0692± 0.0188	0.1017± 0.0274	0.0779± 0.037	0.0605± 0.0285	0.0553± 0.0391	0.0953± 0.0789	0.0427± 0.0238	0.0646± 0.0488
(+/-)-trans-Nerolidol	S	I	1563	0±0	0±0	0±0	0±0	0±0	0±0	0.1681± 0.0479	0.0953± 0.0363	0.2317± 0.0883	0.2151± 0.1089	0.0919± 0.0373	0.1456± 0.0626
trans,trans-Farnesal	S	C	1742	0.0337± 0.0073	0.0336± 0.0087	0.0248± 0.0061	0.0391± 0.0076	0.0341± 0.0053	0.0495± 0.0103	0±0	0±0	0±0	0±0	0±0	0±0
Other															
Isophytol (alcohol)	S	I	1946	0±0	0±0	0±0	0±0	0±0	0±0	0.0349± 0.0151	0.0161± 0.006	0.0249± 0.0104	0.0182± 0.0059	0.0072± 0.0034	0.0227± 0.0094
Unknowns															
Unknown 1		I	1093	0±0	0±0	0±0	0±0	0±0	0±0	0.0372± 0.0119	0.0247± 0.0129	0.0422± 0.0137	0.0225± 0.0103	0.0201± 0.0093	0.0247± 0.0093
Unknown 2		C,I	1115	0.4607± 0.096	0.4521± 0.0882	0.436±0 .0866	0.5039± 0.0619	0.553±0 .1066	0.7154± 0.1525	1.6825± 0.4125	1.2315± 0.4085	1.6996± 0.4911	1.2759± 0.4241	1.0382± 0.3311	1.2834± 0.385
Unknown 3		I	1122	0±0	0±0	0±0	0±0	0±0	0±0	0.0157± 0.0038	0.0103± 0.0049	0.0114± 0.0049	0.0119± 0.0052	0.0096± 0.0043	0.0096± 0.0043
Unknown 4		I	1212	0±0	0±0	0±0	0±0	0±0	0±0	0.0461± 0.0114	0.0549± 0.0175	0.0415± 0.0102	0.0385± 0.0114	0.0287± 0.007	0.0414± 0.0115
Unknown 5		I	1324	0±0	0±0	0±0	0±0	0±0	0±0	0.0316± 0.0129	0.0134± 0.0081	0.0215± 0.0077	0.021± 0.0106	0.0153± 0.0077	0.0177± 0.0065
Unknown 6		I	1386	0±0	0±0	0±0	0±0	0±0	0±0	0.0331± 0.0135	0.0145± 0.0095	0.0551± 0.0328	0.0194± 0.008	0.0155± 0.0116	0.0168± 0.0069
Unknown 7		I	1444	0±0	0±0	0±0	0±0	0±0	0±0	0.0145± 0.0051	0.0084± 0.0043	0.0219± 0.0099	0.0119± 0.0042	0.0123± 0.0054	0.0127± 0.0044
Unknown 8		I	1550	0±0	0±0	0±0	0±0	0±0	0±0	0.0216± 0.0091	0.0107± 0.0064	0.0359± 0.0226	0.0149± 0.0057	0.0104± 0.006	0.0141± 0.0066
Unknown 9		C,I	1579	1.1025± 0.2858	1.0374± 0.354	0.804±0 .2324	1.2077± 0.2221	1.1852± 0.2774	1.8864± 0.5185	0.8649± 0.2843	0.3642± 0.0802	0.672± 0.2174	0.2797± 0.0768	0.1735± 0.0525	0.4626± 0.2475

Table 4.3, continued

Unknown 10		I	1704	0±0	0±0	0±0	0±0	0±0	0±0	0.0165± 0.0075	0.0108± 0.0062	0.0237± 0.0077	0.0113± 0.006	0.0111±0 .0048	0.0228± 0.0078
Unknown 11		I	1853	0±0	0±0	0±0	0±0	0±0	0±0	0.0247± 0.01	0.0151± 0.0093	0.0199± 0.011	0.0127± 0.005	0.011±0. 0066	0.0149± 0.0058
Unknown 12		I	1917	0±0	0±0	0±0	0±0	0±0	0±0	0.0113± 0.0035	0.0072± 0.0024	0.0109± 0.0033	0.0095± 0.0031	0.0047±0 .0014	0.0077± 0.002
Unknown 13		I	2029	0±0	0±0	0±0	0±0	0±0	0±0	0.0619± 0.0256	0.0147± 0.0068	0.0306± 0.0125	0.0331± 0.0113	0.0081±0 .0035	0.0313± 0.0186

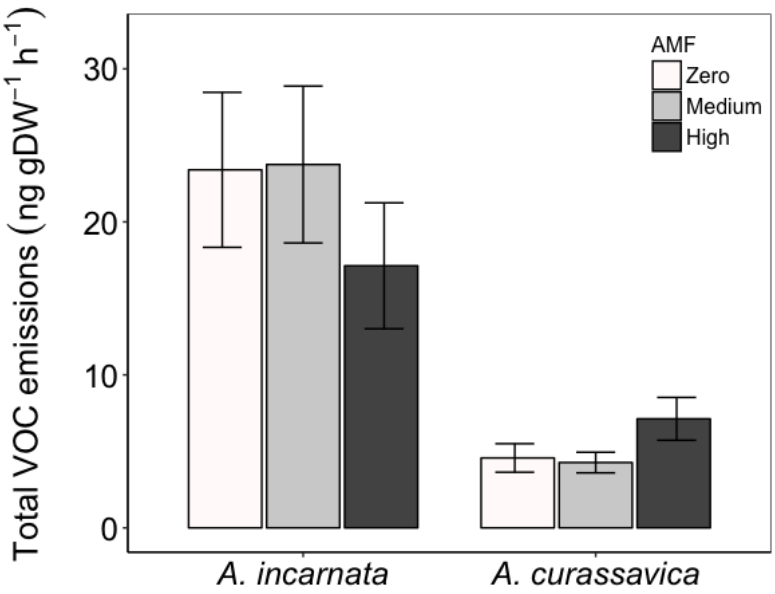

Figure 4.1. Effects of AMF inoculum availability on total VOC emissions by two species of milkweed. Samples sizes are 24 plants per plant species x AMF treatment. Bars display the mean ± 1 SE.

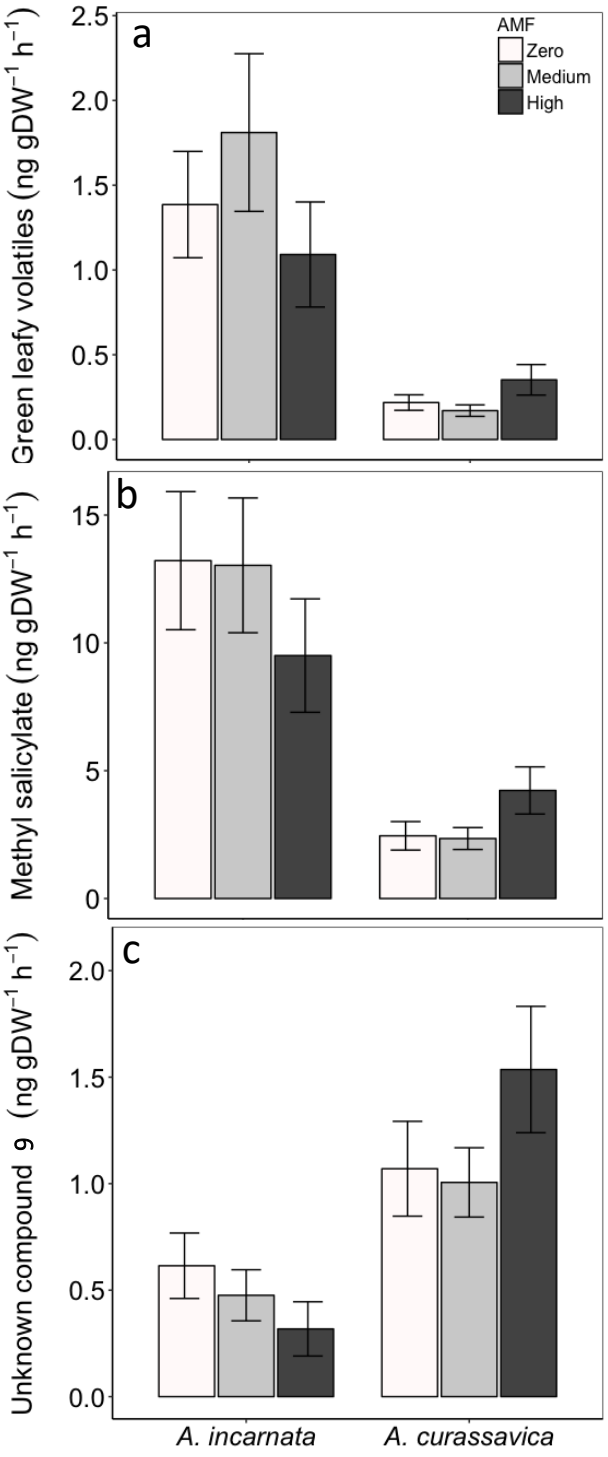

Figure 4.2. Effects of AMF inoculum availability on the emission of a) green leafy volatiles (GLVs), b) methyl salicylate, and c) unknown compound 9 by two milkweed species. Samples sizes are 24 plants per treatment combination. Bars display the mean ± 1 SE.

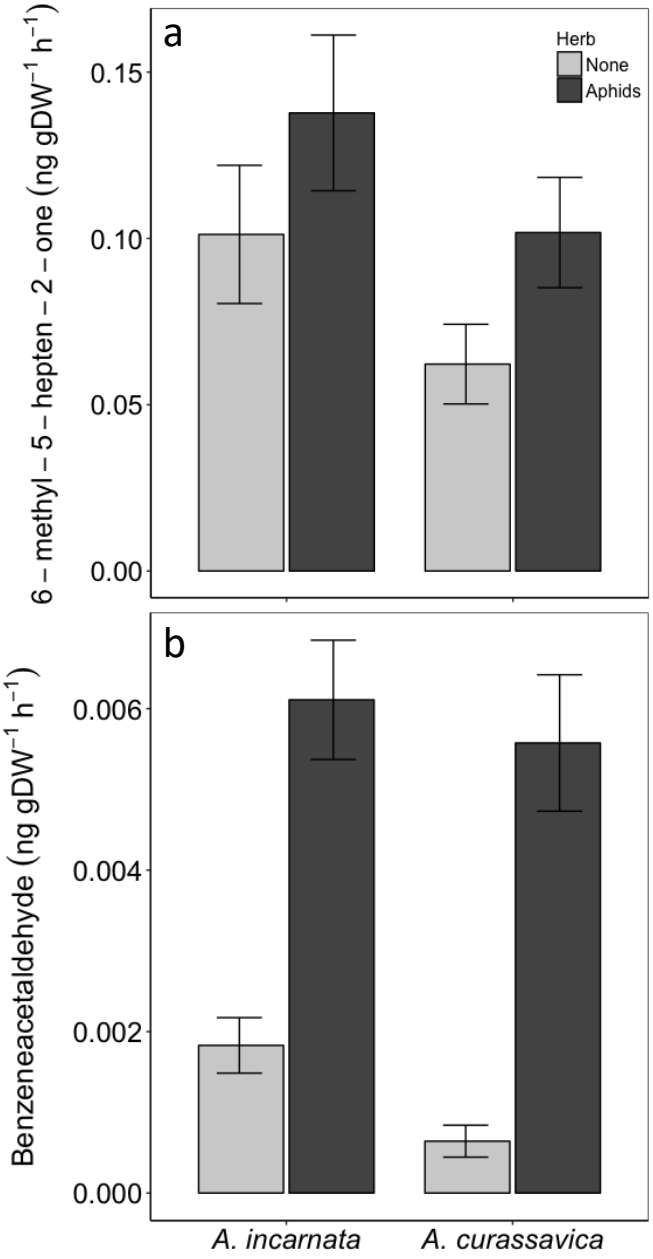

Figure 4.3. Effects of aphid feeding on the emission of a) 6-methyl-5-hepten-2-one and b) benzeneacetaldehyde by two milkweed species. Samples sizes are 36 plants per treatment combination. Bars display the mean ± 1 SE.

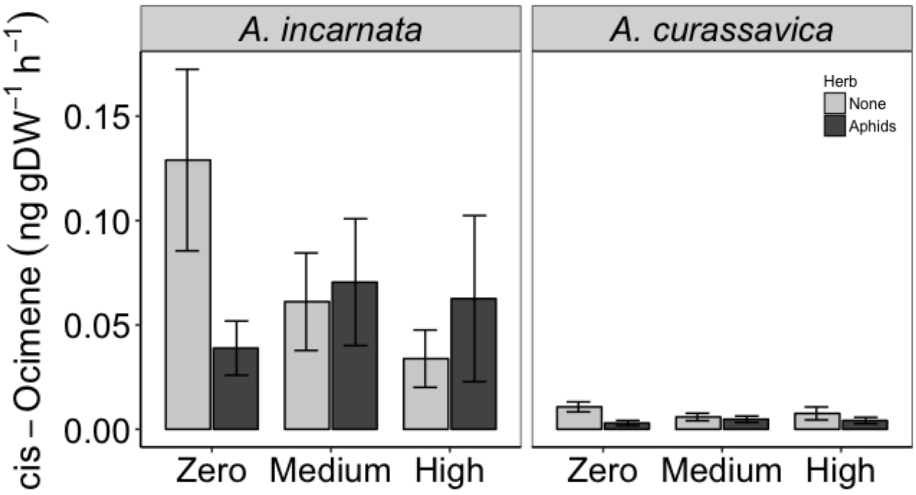

Figure 4.4. Effects of AMF inoculum availability and aphid feeding on cis-ocimene emissions by two milkweed species. Samples sizes are 12 plants per treatment combination. Bars display the mean ± 1 SE.

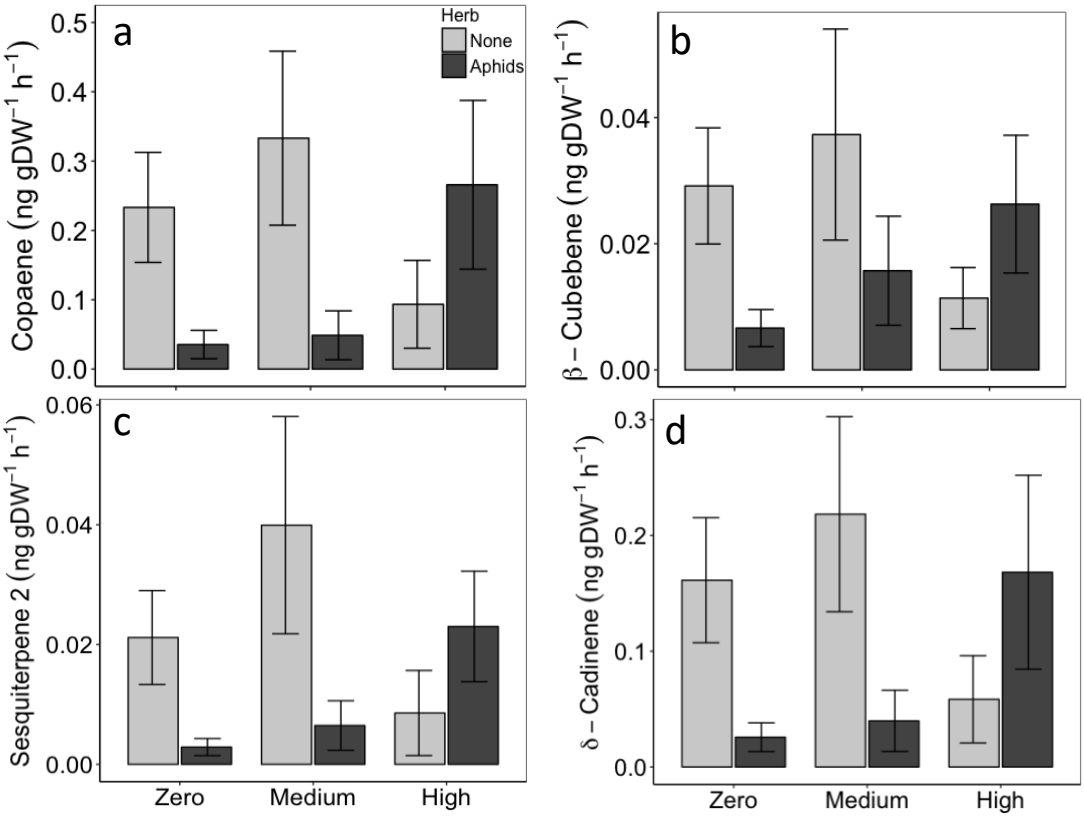

Figure 4.5. Effects of AMF inoculum availability and aphid feeding on the emissions of a) copaene, b) beta-cubebene, c) sesquiterpene 2, and d) delta-cadinene by *A. incarnata*. Samples sizes are 12 plants per treatment combination. Bars display the mean ± 1 SE.

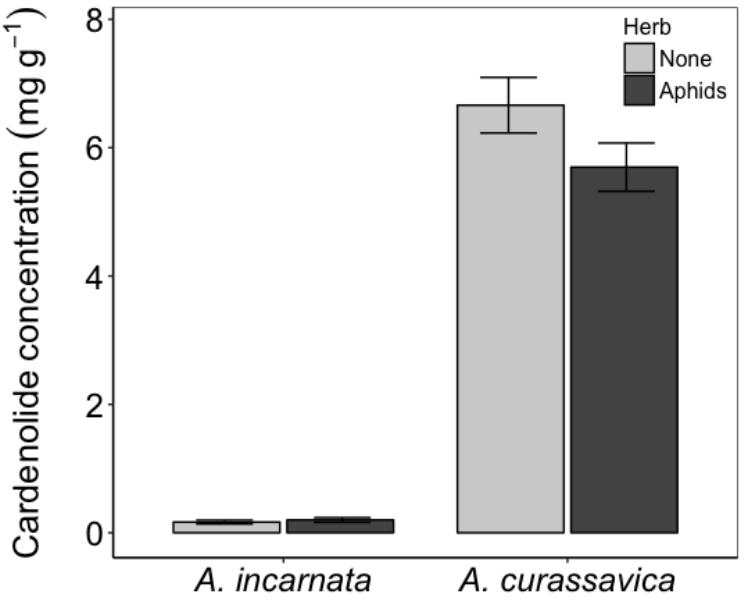

Figure 4.6. Effects of aphid feeding on the foliar cardenolide concentrations of two milkweed species. Samples sizes are 36 plants per treatment combination. Bars display the mean ± 1 SE.

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Chapter V

Arbuscular mycorrhizal fungi alter herbivore-predator interactions

Abstract

Multitrophic species interactions are shaped by both top-down and bottom-up forces. Such interactions take place between communities above and below ground, with plants connecting these communities. As a result, organisms below ground, such as arbuscular mycorrhizal fungi (AMF), may affect communities above ground substantially. However, few studies have considered the ecological relevance of belowground organisms on herbivore-natural enemy interactions in the field. We evaluated how AMF influence the natural colonization of milkweed (*Asclepias*) species by herbivores and their natural enemies. In a full factorial design, we inoculated plants of six milkweed (*Asclepias*) species with three levels of AMF inoculum availability. After six weeks of growth in the greenhouse (June), we harvested a subset of plants for nutritive and defensive traits, and grew the remaining plants in the field in a randomized block design. We allowed arthropods to colonize plants naturally and counted them weekly. At the end of the season (October), we harvested plants to determine root colonization by AMF and plant defense traits.

Only oleander aphids (*Aphis nerii*) colonized plants in sufficient numbers to assess the effects of AMF on herbivore colonization. We found that AMF increased the probability of aphid colonization consistently among plant species but, after colonization, altered subsequent aphid abundances differentially among plant species. Following AMF-mediated increases in aphid colonization and abundance, total predator abundances were greatest on plants under high AMF availability, consistently among plant species. Effects of AMF on individual predators were more complex; the probability of spider colonization varied with AMF availability differentially among plant species, irrespective of aphid density. In contrast, aphid midge fly oviposition and predation of aphids on *A. curassavica* plants varied strongly with aphid density and the amount of AMF available to their host plants. Most notably, the per capita mortality rate

imposed by midge flies on aphids varied with AMF availability. Our findings suggest that the availability of AMF in soils may have pervasive effects on herbivore-predator dynamics in the field.

Introduction

Multitrophic species interactions are shaped by a combination of bottom-up forces, such as resource availability, and top-down forces, such as predators and parasites (Hunter and Price 1992, Schmitz et al. 2000). Such interactions can occur among above and belowground organisms, with plants connecting communities above and belowground (van der Putten et al. 2001, Bezemer and van Dam 2005, Erb et al. 2008, Pineda et al. 2010, van Dam and Heil 2011, Johnson et al. 2012, Stam et al. 2014). As a result, organisms belowground can have strong effects on communities aboveground. For instance, root feeders and soil microbes alter the performance of aboveground insect herbivores (Erb et al. 2008, Koricheva et al. 2009, Pineda et al. 2010, Rasmann et al. 2017), and alter the attraction of predators and parasitoids to their herbivore prey (Rasmann and Turlings 2007, Rasmann et al. 2017, Tao et al. 2017). However, few studies have considered the ecological relevance of belowground organisms on herbivore-natural enemy interactions in the field, despite the apparent ubiquity of such interactions (Gange et al. 2003, Ueda et al. 2013, Rasmann et al. 2017, Tao et al. 2017).

Arbuscular mycorrhizal fungi (AMF) take part in one of the most prevalent root-microbe symbioses on land. AMF associate with over 80 percent of plant species, providing nutrients to plants in exchange for sugars (Smith and Read 2008). In doing so, AMF interact with plant defensive signaling pathways (Jung et al. 2012, Cameron et al. 2013, Gutjahr 2014, Bucher et al. 2014) and alter plant nutrient uptake, thereby affecting plant defensive and nutritional traits (Bennett et al. 2009, Vannette et al. 2013, Roger et al. 2013, Schweiger et al. 2014, Schweiger and Müller 2015). The association with AMF is often mutualistic for plants; AMF frequently stimulate plant growth and mitigate abiotic and pathogen stress (Smith and Read 2008). However, the effects of AMF on plant growth and defense range from beneficial to detrimental, depending on the environment (Hoeksema et al. 2010), the identity of plants and AMF (Klironomos 2003, Tao et al. 2016), and the density of AMF inoculum available to plants (Garrido et al. 2010, Vannette and Hunter 2011, 2013).

AMF invoke variable effects on herbivore performance. For instance, AMF often reduce the performance of chewers, but improve the performance of phloem feeders, who may benefit from AMF-mediated increases in nutritive quality while avoiding increases in defensive traits (Hartley and Gange 2009, Koricheva et al. 2009). In addition, AMF-colonized plants can be more attractive to herbivores, due to AMF-mediated changes in plant volatile emissions (Babikova et al. 2014b, 2014a, Simon et al. 2017). Most studies to date have considered only how AMF affect herbivores in the absence of natural enemies, although herbivore population dynamics are often driven by their natural enemies (Turchin et al. 2003). By altering volatile emissions from plants, AMF can increase the attraction of natural enemies to plants (Guerrieri et al. 2004, Schausberger et al. 2012, Babikova et al. 2014b, 2014a), even in the absence of herbivores (Guerrieri et al. 2004). In contrast, by increasing plant size, AMF can reduce the searching efficiency of natural enemies (Gange et al. 2003). Furthermore, AMF often improve the performance of natural enemies, enhancing the population growth of predators (Hoffmann et al., 2011a) and the successful development of parasitoids (Hempel et al. 2009, Bennett et al. 2016). AMF-mediated increases in herbivore performance may even be compensated for by the combination of enhanced predator populations and higher plant tolerance to damage, ultimately leading AMF to benefit their host plants (Hoffmann et al. 2011c). Therefore, to understand the potential impacts of AMF on aboveground communities, we must consider the effects of AMF on multitrophic interactions in the field.

To date, most studies assessing how AMF influence herbivore-predator interactions have been limited to single crop-plant species in greenhouse or laboratory settings. Moreover, no study to date has considered how the availability of AMF inoculum in soil influences multitrophic interactions in the field. The extent of AMF inoculum available to plants varies among habitats (Koide and Mooney 1987) and with land management practices (Lekberg and Koide 2005). AMF availability also varies on small scales, such as centimeters (Wolfe et al. 2007) and meters (Carvalho et al. 2003). Herbivore survival, growth, and sequestration of toxins are altered by AMF availability in soils (Vannette and Hunter 2013, Meier and Hunter 2018), suggesting that AMF availability may shape multitrophic interactions in the field.

Here, we evaluate how AMF influence the colonization of milkweed (*Asclepias*) species by their herbivores and their natural enemies in the field. We hypothesized that AMF would increase colonization by both herbivores and their natural enemies. We did not have specific predictions for the effects of AMF on aphid abundance, as the effects of AMF on plant phenotype and natural enemies may combine to shape aphid abundances. Because the outcomes of AMF-plant associations are specific to the AMF and plant species involved (Gange et al. 2003, Hempel et al. 2009, Grman 2012, Tao et al. 2016, Bennett et al. 2016), we expected the magnitude of these effects to vary with AMF availability differentially among milkweed species.

Plants and Insects

Milkweed species provide an ideal system in which to assess how AMF affect multitrophic interactions, because milkweeds produce a suite of well-characterized resistance traits that show constitutive and AMF-mediated variation (Vannette et al. 2013, Tao et al. 2016, Meier and Hunter 2018). Milkweeds produce cardenolides, bitter tasting steroids that disrupt the functioning of sodium-potassium channels in animal cells (Agrawal et al. 2012). In response to leaf damage, milkweeds exude latex, a sticky isoprene polymer that gums up the mouths of chewing herbivores (Zalucki et al. 2001, Agrawal and Konno 2009). Milkweeds are attacked by a community of specialized herbivores, which can tolerate and sequester milkweed defenses, but are still negatively affected by those defenses (Agrawal 2004, Rasmann et al. 2009, Rasmann and Agrawal 2011). Both milkweed herbivore performance and toxin sequestration vary with the amount of AMF available to plants (Vannette and Hunter 2013, Meier and Hunter 2018), indicating that AMF may affect multitrophic interactions in the field. While milkweeds are naturally colonized by a diversity of herbivores, only oleander aphids (*Aphis nerii*) colonized plants in this study in sufficient numbers to assess the effects of AMF on herbivore colonization. In the field, *A. nerii* are killed by a suite of generalist predators and parasitoids, including lacewings (Neuroptera), syrphids (Diptera), coccinelids (Coleoptera), spiders (Araneae), aphid midge flies (*Aphidoletes aphidimyza*, Diptera), and parasitoid wasps (Hymenoptera) (Malcolm 1992, Helms et al. 2004, Mooney et al. 2010, Mohl et al. 2016).

We used six North American milkweed species (*Asclepias curassavica*, *A. incarnata*, *A. speciosa*, *A. verticillata*, *A. sullivantii*, *A. syriaca*) that show constitutive and AMF-mediated

variation in defenses and nutritive quality (Vannette et al. 2013, Tao et al. 2016). *Asclepias incarnata* and *A. syriaca* seeds were collected from naturally occurring populations in Emmet County, MI. *Asclepias speciosa*, *A. verticillata*, and *A. sullivantii* seeds were purchased from Prairie Moon Nursery (Winona, MN, USA, ww.prairiemoon.com) and *A. curassavica* seeds were purchased from Victory Seeds (Molalla OR, www.victoryseeds.com). We obtained our AMF inoculum from Mycorrhizal Applications (Grants Pass, OR, USA), which is advertised to contain four AMF species including *Rhizophagus intraradices*, *Funneliformis mosseae*, *Glomus aggregatum*, and *Claroideoglomus etunicatum* (33 spores of each AMF species per gram inoculum, www.plant-success.com). However, we later found this mix to contain only *Funneliformis mosseae* (Meier and Hunter 2018). Milkweed species grow in habitats that host a diversity of AMF taxa (Öpik et al. 2006), and can form associations with these cosmopolitan AMF species in natural and experimental populations (Vannette et al. 2013, Tao et al. 2016, Meier and Hunter 2018), although the frequency of such interactions is unknown.

Experimental Protocols

Seeds of all plant species were cold stratified for 6 weeks at 4°C (except the tropical *A. curassavica*), surface sterilized in 5% bleach, and germinated at room temperature. We planted individual seedlings into conical deepots (D40H, Steuwe and Sons, Inc., Corvalis, OR, USA) filled with 600 ml of a mix of autoclaved soil (Metro-Mix 360, MetroMix Sun Gro Horticulture Canada CM Ltd., Vancouver, BC, Canada) and sand (5:3) containing AMF inoculum. We homogenized 4.20 g of autoclaved AMF inoculum (zero treatment), 1.20 g live and 3.00 g autoclaved inoculum (medium treatment), or 4.20 g live inoculum (high treatment) in 200 ml of autoclaved soil and sand, which was placed between 400 ml autoclaved soil and sand. We returned the natural bacterial community to the soil by adding 20 ml of a suspension of 100 ml soil in 1L deionized water filtered through an ultra-fine sieve (32 µm) to remove AMF hyphae and spores. We grew plants in the greenhouse under 15L:9D, watered plants *ad libitum*, and fertilized them weekly with 30 ml of 15-0-15 (N-P-K, 567 ppm) dark weather fertilizer (JR Peters Inc., Allentown, PA).

After six weeks of growth (June 2015), we harvested a subset of plants (10 of each treatment combination, N=180) to measure initial plant traits and AMF colonization (below).

Plants were harvested in two blocks, each separated by one day, with treatments equally represented within blocks. Concurrently, fifteen to twenty-two replicates of each treatment combination were placed in a randomized block design with four blocks in a mowed field at the Matthaei Botanical Gardens (Ann Arbor, MI). Sample sizes ranged from 15-22 plants per treatment due to variation in germination success among plant species. Four to six replicates of each AMF x plant species combination were placed in each of the four blocks. Plants were elevated above the soil surface in 24-inch plant props (Luster Leaf, www.lusterleaf.com) to prevent colonization by natural AMF. Each week, we tallied phytophagous arthropods and assigned them to general taxonomic groups. However, only *Aphis nerii* colonized our plants in high enough numbers to analyze their abundance, and aphid colonization did not commence until August 13, 2015. Active, predaceous arthropods and their eggs were tallied and assigned to general taxonomic groups, including spiders, coccinellids, lacewings, aphid midge flies, and syrphids. We counted arthropods weekly until October 1, 2015 (8 weeks) as plants started to senesce. We measured plant height biweekly from June 1, 2015 till October 1, 2015.

To measure foliar traits from the plants harvested in June, we punched three fresh leaf disks from each leaf of the sixth leaf pair (six hole punches, 424 mm^2 total) of *A. incarnata*, *A. curassavica*, and *A. syriaca* plants, placed the disks in 1 mL of methanol, and stored them at -10°C until cardenolide analysis. An equivalent amount of leaf tissue was taken from *A. verticillata*, *A. speciosa*, and *A. sullivantii*; the leaves of these species were too thin to collect hole punches. Latex that exuded from leaves was collected on pre-weighed cellulose disks, dried at 50°C, and weighed. Six additional leaf disks, or equivalent pieces, were taken from the same leaves, weighed, stored in glassine envelopes, and dried at 50°C. Samples were re-weighed to provide estimates of leaf water content and sample mass for cardenolide analysis.

Additional leaves from neighboring leaf pairs were removed from each plant and dried at 50°C for subsequent carbon (C), nitrogen (N), and phosphorous (P) analyses. Remaining plant material was dried at 50°C and weighed to measure aboveground biomass after correcting for foliar tissue removed for chemistry sampling. We assessed the C and N concentrations of foliar tissues with a TruMac elemental analyzer (Leco Corporation, St. Joseph, MI, 49085, USA). P concentrations of foliar samples were determined by dry combusting ground samples in a muffle

furnace at 550°C overnight, followed by persulfate-acid digestion and analysis by an autoanalyzer (Alpkem FS3000, EZkem, Hood River, OR, USA). We calculated P concentrations of samples from a potassium phosphate standard curve and assessed quality control with NIST apple leaf standard analyzed with all samples.

After washing roots in deionized water, we stored 150 mg of 1 cm pieces of fresh fine root tissue in 60% ethanol at 4°C until we could quantify AMF colonization. We also took 400 mg of fresh fine root, dried it at 50°C, and reweighed it to calculate the wet weight/dry weight ratios from which to estimate the dry mass of the subsample taken to quantify AMF colonization. We dried the remaining root tissue at 50°C and weighed its contribution to total root biomass. To quantify AMF colonization, we cleared roots with 10% KOH for 10 min, acidified them using 2% HCl, and stained them in 0.05% trypan blue in 1:1:1 water:glycerol:lactic acid (Vannette and Hunter 2011). We mounted stained roots on slides and scored AMF colonization using the magnified gridline intersect method (McGonigle et al. 1990) with a Nikon compound microscope (Melville, NY, USA). A root intersection was considered colonized if hyphae, arbuscules, or vesicles were present. At least 100 root intersections were analyzed per plant.

To assess how AMF affected cardenolide expression in milkweeds, we quantified foliar cardenolide concentrations following established methods (Zehnder and Hunter 2007, Meier and Hunter 2018). In brief, cardenolides were extracted from foliar samples in methanol. Samples were then separated by high performance liquid chromatography (UPLC; Waters Inc., Milford, MA, USA) using a Luna 2.5 µm C18(2) column (Phenomenex Inc., Torrance, CA, USA) with digitoxin as an internal standard. Peaks with symmetrical absorbance between 218 and 222 nm were quantified as cardenolides and total cardenolide concentrations were calculated as the sum of individual peaks. In addition, we calculated cardenolide diversity using Shannon's index and a cardenolide polarity index (the relative representation of lipophilic cardenolides) by summing the relative peak areas multiplied by each peaks' retention time (Rasmann and Agrawal 2011, Sternberg et al. 2012). Evidence suggests that more diverse and lipophilic cardenolides are more toxic than are less diverse or more polar mixes (Fordyce and Malcolm 2000, Zehnder and Hunter 2007, Sternberg et al. 2012).

At the end of the growing season (October 1, 2015), we harvested plants to measure the proportion of roots colonized by AMF and the cardenolide concentrations of leaves, as described above. Aphids and their natural enemies continue to feed on milkweed stems during and after leaf senescence. Because we wanted to evaluate aphid-predator dynamics until the end of their local life cycles, many plants had begun leaf senescence by the time of the final harvest. As a consequence, there was not sufficient leaf material to provide replicate foliar samples from either *A. syriaca* or *A. sullivantii* (Table 5.4). Therefore, these plant species were excluded from late-season cardenolide analyses. In addition, there was not enough foliage remaining to conduct late-season nutrient analyses for any milkweed species.

Data Analyses

General Methods

In all the analyses that follow, residuals were checked for normality and homogeneity of variance. Data were natural log-transformed when necessary. All statistical analyses were performed in SAS 9.4 (SAS Institute, Cary, NC, USA) using the function GLIMMIX for generalized linear mixed models and MIXED for general linear mixed models. Sample sizes were 10 plants per treatment for all plant traits except nutrient analyses in the early season (June) harvest; not all plants had sufficient foliar material for nutrient analyses due to small plant biomasses (Table 5.2). Sample sizes for insect field data ranged from 15-22 plants per treatment, as explained above. Samples sizes for late-season plant traits varied greatly due to senescence of leaves at the end of the season (Table 5.4).

Herbivores

Due to low rates of aphid colonization on most milkweed species throughout the season (Fig. 5.1), we were unable to assess aphid dynamics over time. Therefore, we calculated cumulative aphid numbers on each individual plant by summing counts beginning the first week that aphids colonized plants (August 13, 2015) until the end of the growing season (October 1, 2015). Because many plants were never colonized by aphids (62%), we first compared the effects of AMF inoculum availability and milkweed identity on the probability of aphid colonization using a generalized linear mixed model with a binomial distribution and logit link function. Whether or not a plant was ever colonized by aphids was the binary dependent

variable, and milkweed species, AMF inoculum availability, and their interaction were fixed effects. Block was designated as a random effect. Next, using only those plants that were colonized by aphids, we evaluated the effects of AMF availability and milkweed species on cumulative aphid abundance. For this analysis, we used a generalized linear mixed model with a Poisson distribution and log link function. Aphid abundance was the dependent variable, milkweed species, AMF inoculum availability, and their interactions were fixed factors, and block was a random effect. To consider whether differences in aphid colonization and abundance resulted from variation in plant height, we repeated the analyses above for aphid presence and abundance, but included plant height as a covariate. We calculated plant height as the average height of individual plants from August 10, 2018 till the end of the season. We reasoned that, if any effects of AMF and milkweed species on aphid abundances detected in the first analysis remained after adding height to the model, then effects of AMF and milkweed species on aphid population sizes could not be explained by plant size alone.

Predators

Because there were also low predator counts each week, we calculated the cumulative numbers of predators on each plant over the sampling period by summing together counts of all aphid midge (*Aphidoletes aphidimyza*) larvae, coccinelid adults and larvae, lacewing larvae, syrphid larvae, and spiders from when aphids first colonized plants (August 13, 2015) until the end of the growing season (October 1, 2015; 8 weeks of counts). We did not include predator eggs in our calculation. We evaluated the effects of AMF availability and milkweed identity on the cumulative abundance of predators on plants using a generalized linear mixed model with a negative binomial distribution and log link function. Predator abundance was the dependent variable, AMF availability, milkweed species, and their interactions were fixed factors, and block was a random effect. To evaluate whether variation in predator abundance was mediated by aphid abundance, we repeated the above analysis, but added aphid abundance to the model as a covariate. Again, we reasoned that, if any effects of AMF and milkweed species on predators detected in the first analysis remained after adding aphid abundance to the model, then effects of AMF and milkweed species on predator population sizes could not be explained by aphid densities alone.

Because spiders and midge flies (*Aphidoletes aphidimyza*) made up 81% of all predator counts, we evaluated the effects of AMF availability and milkweed identity separately for these predator groups. Spiders never colonized *A. speciosa* plants under zero or low AMF availability, so we excluded *A. speciosa* from analyses of spider abundance. Because many individual plants never hosted spiders (43%), we first evaluated the effects of AMF availability and milkweed identity on the probability that spiders would colonize plants using a generalized linear mixed model with a binomial distribution and logit link function. Next, using only those plants that hosted spiders, we assessed how AMF availability and milkweed identity influenced the abundance of spiders using a generalized linear mixed model with a Poisson distribution and log link function. In both analyses, spider presence or abundance was the dependent variable, AMF availability, milkweed species, and their interaction were fixed effects, and block was a random effect. To elucidate whether variation in spiders among treatment groups was mediated primarily by aphid abundance, we repeated these analyses with aphid density as a covariate.

Finally, we assessed how aphid midge fly oviposition and predation of aphids varied among AMF treatments on *A. curassavica*; no other milkweed species hosted a sufficient abundance of midge flies for analyses. Aphid midge fly eggs and larvae were only present on plants colonized by aphids, so we included only these plants in the following analyses. Aphids killed by aphid midge flies have characteristic black shells that persist on plants for approximately one week. We summed these dead aphids as a metric of predation by aphid midge larvae. We assessed effects of AMF availability on aphid midge fly oviposition (cumulative number of eggs), midge larva abundance (cumulative number of larvae), and predation (cumulative number of dead aphids) using generalized linear mixed models with a Poisson distribution and log link function. Aphid abundance, milkweed species, AMF treatment, and their interactions were fixed effects. Block was designated as a random effect.

Plant traits

To evaluate effects of AMF availability and milkweed identity on average plant height and plant traits early (June, 6 week-old plants) and late in the season (October, 5 month-old plants), we used general linear mixed models, with block as a random effect. Each plant trait was the dependent variable, and milkweed species, AMF treatment, and their interaction were fixed

effects. *A. incarnata* produced no cardenolides at the beginning of the season, and was therefore excluded from analyses of June cardenolides. At the end of the season (October), only one individual plant of *A. syriaca* and *A. sullivantii* without AMF still had leaves, so they were excluded from October cardenolide analyses.

Results

AMF colonization

Our AMF treatments were successful in generating variation in AMF colonization among treatments. Early in the season (June, 6-week old plants), plants without AMF had no colonization, plants under medium AMF availability had 10% (±1.6) root colonization, and plants under high AMF availability had 21% (±1.8) root colonization (AMF $F_{2,161}$=60.72, P<0.0001), although the extent of increase in root colonization under medium and high AMF availability varied among plant species (AMF x plant species $F_{10,161}$=1.97, P=0.0394; Tables 5.1,5.2). By the end of the season (October, 5-month old plants), plants in our zero AMF treatment had an average of 2% (± 0.1) colonization, and plants under medium and high AMF availability had 43% (±1.9) and 52% (± 1.4) colonization, respectively, across all plant species (AMF $F_{2,319}$=422.37, P<0.0001; Tables 5.3,5.4). AMF colonization did not vary among species (Tables 5.3,5.4).

Herbivores

Across all plant species, aphids were, on average, 35 and 48% more likely to colonize plants under medium and high AMF availability, respectively, than plants without AMF (AMF $F_{2,313}$=2.95, P=0.0537, AMF x plant species $F_{10,313}$=0.57, P=0.8417; Fig. 5.1a). In addition, aphids were most likely to colonize *A. curassavica* plants, and least likely to colonize *A. speciosa* or *A. verticillata* plants (Plant species $F_{5,313}$ = 6.62, P<0.0001; Fig. 5.1b). Upon colonizing plants, aphids reached their greatest abundances on plants inoculated with AMF (AMF $F_{2,104}$=72.82, P<0.0001; Fig. 5.2), although the extent of increase varied among plant species (AMF x plant species $F_{10,104}$=111.46, P<0.0001, Fig. 5.2). For example, aphid populations were 74 and 134% greater on *A. curassavica* and *A. syriaca* plants, respectively, under medium AMF availability than without AMF. *A. curassavica* and *A. syriaca* plants under high AMF availability hosted intermediate aphid abundances. Aphid abundances were 21.8 times greater on *A. speciosa* plants

under medium and high AMF availability than on plants without AMF. In contrast, AMF did not affect aphid abundances on *A. incarnata*, *A. sullivantii*, or *A. verticillata* (Fig. 5.2). Aphid abundances also varied 4-fold among plant species, being greatest on *A. curassavica* and least on *A. sullivantii* (Plant species $F_{5,104}$=724.30, P<0.0001; Fig. 5.2)

While average plant height varied strongly among plant species (Plant species $F_{5,320}$=347.93, P<0.0001, Table 5.4), AMF did not affect plant height (AMF $F_{2,320}$=0.42, P=0.6575, AMF x plant species $F_{10,320}$=0.20 P=0.9965, Table 5.4). Moreover, while the probability of aphid colonization and aphid abundance increased with plant height (Aphid colonization $F_{1,312}$=8.69, P=0.0034; abundance $F_{1,103}$=646.26, P<0.0001), the probability of aphid colonization, in models containing height as a covariate, still varied among AMF treatments (AMF $F_{2,312}$=3.04, P=0.0491) and plant species (Plant species $F_{5,312}$=7.21, P<0.0001). Similarly, aphid abundance still varied with AMF availability differentially among plant species in models containing height as a covariate (AMF x plant species $F_{10,103}$=115.94, P<0.0001). Accordingly, significant effects of AMF availability on the probability of aphid colonization and aphid abundance cannot be driven by increases in plant height.

Predators

Across all plant species, plants under high AMF availability hosted, on average, 45% more predators than did plants under medium AMF availability or those without AMF (AMF $F_{2,313}$=4.45, P=0.0124, Fig. 5.3a). In addition, predator abundance varied 6-fold among plant species, with the greatest number of predators on *A. curassavica* and the least on *A. speciosa* (Plant species $F_{5,313}$=7.96, P<0.0001, Fig. 5.3b). AMF-mediated increases in predator abundances were driven by increases in aphid abundance (Aphids $F_{1,312}$=116.52, P<0.0001) such that effects of AMF on predator numbers were no longer significant in models that included aphid abundance as a covariate (AMF $F_{2,312}$=2.22, P=0.1108). However, differences in predator abundance among plant species persisted once accounting for aphid abundance (Plant species $F_{5,312}$=8.40, P<0.0001).

In contrast to the consistent, positive effects of AMF on total predator abundance among plant species, AMF altered the probability of spider colonization differentially among milkweed

species (AMF x plant species $F_{8,263}$=2.03, P=0.0437; Fig. 5.4). Spiders were 2.8 times more likely to colonize *A. sullivantii* plants under medium AMF availability than plants under high AMF availability or without AMF. In contrast, spiders were 19 and 37% less likely to colonize *A. incarnata* and *A. verticillata* plants, respectively, under medium AMF availability than plants under high AMF availability or without AMF. In addition, spiders were 2.2 times more likely to colonize *A. syriaca* plants without AMF than plants under medium or high AMF availability. AMF did not affect the probability of spider colonization of *A. curassavica* (Fig. 5.4). Spider presence also varied among plant species, being greatest on *A. incarnata* and *A. curassavica* and least on *A. sullivantii* (Plant species $F_{4,263}$=5.35, P=0.0004; Fig. 5.4). Spider presence was unaffected by aphid abundance (Aphids $F_{1,262}$=0.02, P=0.8981). In addition, the abundances that spiders reached on the plants that they colonized did not vary with AMF availability, plant species, or aphid abundance (AMF $F_{2,143}$=0.28, P=0.7550; Plant species $F_{4,143}$=0.71, P=0.5837; AMF*plant species $F_{8,143}$=1.05, P=0.4002; Aphids $F_{1,142}$=0.44, P=0.5066).

Importantly, aphid midge flies laid the most eggs relative to aphid number on plants without AMF, and the least eggs relative to aphid number on plants under high AMF availability (Aphids x AMF: $F_{2,29}$=5.85, P=0.0073, Fig. 5.5a). However, while the abundance of midge larvae increased with aphid number (Aphids $F_{1,29}$=6.63, P=0.0154), midge larva abundance was not affected by AMF availability (AMF $F_{2,29}$=1.18, P=0.3209; Aphids x AMF $F_{2,29}$=0.79, P=0.4655). In contrast, the rate of mortality by aphid midge flies, relative to aphid abundance, was greatest on plants under high AMF availability and least under medium AMF availability (Aphids x AMF: $F_{2,29}$=73.59, P<0.0001; Fig. 5.5b). In addition, 108% more aphids were killed by midge flies on medium and high AMF plants than on plants without AMF (AMF $F_{2,29}$=60.73, P<0.0001).

Plant traits

Early in the season (June), AMF increased aboveground biomasses of three of the six milkweed species (*A. curassavica, A. speciosa, A. syriaca*) (AMF x Plant species $F_{10,161}$=2.33, P=0.0137; Table 5.2), and increased belowground biomasses of all plant species (AMF $F_{2,160}$=3.45, P=0.034; Table 5.2). In addition, across all plant species, plant height increased by an average of 13% under high AMF compared to plants under medium or zero AMF availability

(AMF $F_{2,161}$=3.51, P=0.0322; Table 5.2). Inoculation with medium AMF availability also increased foliar water content by 7% when compared with other AMF treatments, consistently among plant species (AMF $F_{2,161}$=6.42, P=0.0021; Table 5.2). While AMF did not affect total cardenolide concentrations or their polarity, plants under high AMF availability produced a 26% greater diversity of cardenolides than did plants under medium AMF availability; plants without AMF produced an intermediate diversity of cardenolides (AMF $F_{2,112}$=3.91 P=0.0228; Table 5.2). Foliar cardenolide concentrations, diversity, and polarity varied strongly among plant species; cardenolides were most concentrated, most diverse, and most lipophilic in *A. curassavica* and least concentrated, least diverse, and most polar in *A. verticillata* (Tables 5.1,5.2). In addition, leaf number, foliar C, N, and P concentrations, and latex exudation, varied among plant species, but were unaffected by AMF availability (Tables 5.1,5.2)

Although AMF did not affect cardenolide concentrations early in the growing season (above), AMF altered cardenolide concentrations in a plant-species specific manner by the end of the season (October; AMF x plant species $F_{6,179}$=2.52, P=0.0229; Table 5.4). *A. curassavica* plants under medium AMF availability produced 35% greater concentrations of cardenolides than plants under high AMF availability, and plants without AMF produced an intermediate concentration of cardenolides (Table 5.4). In contrast, *A. incarnata* and *A. speciosa* plants under medium and high AMF availability produced 87 and 77% lower concentrations of cardenolides, respectively, than did plants without AMF. AMF did not affect cardenolide concentrations in *A. verticillata*. In addition, plants inoculated with AMF had a lower diversity of more polar cardenolides, consistently among plant species (AMF: Diversity $F_{2,151}$=5.77, P=0.0038; Polarity $F_{2,151}$=4.04, P=0.0195; Tables 5.3,5.4). Finally, while AMF affected plant biomass above and belowground at the beginning of the season, AMF had no effect on plant biomass above or belowground at the end of the season (Tables 5.3,5.4).

Discussion

We found that AMF increase the probability of aphid colonization consistently among plant species (Fig. 5.1) but alter aphid abundances differentially among plant species (Fig. 5.2). Following AMF-mediated increases in aphid colonization and abundance, total predator abundances are greatest on plants under high AMF availability, consistently among plant species

(Fig. 5.3). Effects of AMF on individual predators are more complicated; the probability of spider colonization varies with AMF availability differentially among plant species, irrespective of aphid density (Fig. 5.4). In contrast, aphid midge fly oviposition and predation of aphids on *A. curassavica* plants vary strongly with aphid density and the amount of AMF available to their host plants (Fig. 5.5). Our findings suggest that the availability of AMF in soils may have pervasive effects on herbivore-predator dynamics in the field.

The higher rates of aphid colonization that we observed on milkweed plants inoculated with AMF could not be explained by AMF-mediated increases in plant height. The colonization of plants by aphids represents a complex combination of long- and short-term attraction, and acceptance after alighting (Kennedy et al. 1961, Kennedy 1986, Powell et al. 2006) High levels of AMF in milkweed roots may therefore enhance certain cues used by *A. nerii* during milkweed colonization. For example, AMF have strong effects on constitutive plant volatiles, including terpenes and green leafy volatiles (Rapparini et al. 2008, Fontana et al. 2009, Leitner et al. 2010, Asensio et al. 2012, Schausberger et al. 2012, Babikova et al. 2014b, 2014a, Shrivastava et al. 2015), which are known to influence herbivore attraction (Bruce et al. 2005, Arimura et al. 2009, Turlings and Erb 2018). We have found AMF to alter these classes of compounds in *A. incarnata* and *A. curassavica* (A. R. Meier, unpublished data), indicating that differential *A. nerii* colonization among AMF treatments may indeed be driven by AMF-induced changes in volatiles. Consistent with this hypothesis, volatiles of AMF-colonized *Vicia faba* plants are more attractive to aphids than are the volatiles of plants without AMF (Babikova et al. 2014b, 2014a).

Despite consistent positive effects of AMF on aphid colonization, we observed substantial variation in the effects of AMF on aphid abundance among milkweed species. The AMF-mediated increases in aphid abundance that we observed on *A. curassavica*, *A. speciosa*, and *A. syriaca* are consistent with the increased aphid abundances found on mycorrhizal soybean plants (Ueda et al. 2013). However, aphid abundances were unaffected by AMF on *A. incarnata*, *A. sullivantii*, or *A. verticillata* (Fig. 5.2). Milkweed-specific effects of AMF on aphid abundance may result from differential effects of AMF availability on plant phenotype, including nutritive and defensive traits (Grman 2012, Barber et al. 2013, Anacker et al. 2014, Tao et al. 2016). Whether aphids benefit (Gange and West 1994, Gange et al. 1999, 2002, Koricheva et al. 2009,

Babikova et al. 2014a, b, Simon et al. 2017), are unaffected (Wurst et al. 2004, Colella et al. 2014, Bennett et al. 2016), or are negatively affected by AMF colonization of their host plants (Gehring and Whitham 2002, Hempel et al. 2009, Abdelkarim et al. 2011) depends on the species-specific effects of AMF on plant phenotype. Furthermore, AMF alter plant phenotype differently depending on plant age and the developmental stage the symbiosis, with consequences for herbivore performance (Tomczak and Müller 2017). Therefore, although AMF had minimal effects on milkweed nutritive and defensive traits early in the season (Tables 5.1,5.2), AMF may have altered milkweed traits later in the season, leading to the differences in aphid abundance that we observed. Indeed, some of the AMF-mediated changes in plant phenotype that we observed were limited to late-season samples. For instance, AMF altered cardenolide concentrations late, but not early, in the growing season in a species-specific manner; cardenolides are well known to affect *A. nerii* population growth (Agrawal 2004, Zehnder and Hunter 2007, de Roode et al. 2011), Unfortunately, because our plants were relatively small, we were unable to sample plant tissue mid-season when aphids first colonized plants. However, future work should assess plant traits throughout the season to evaluate how effects of AMF on plant phenotype may drive aphid abundances.

In addition to bottom-up effects of plant phenotype on aphids, top-down effects of predators may have contributed to the variation in aphid abundances that we measured among milkweeds. Under greenhouse conditions in the absence of predators, *Aphis nerii* population growth rates and individual masses are greatest on plants under high AMF availability, least on plants under medium AMF availability, and intermediate on plants without AMF, consistently among milkweed species (Meier and Hunter 2018). Notably, we did not find this nonlinear pattern of aphid abundance with AMF availability on any milkweed species under field conditions in the presence of predators (Fig. 5.2). Furthermore, aphid populations persisted, on average, for only three weeks in the field before being driven extinct, suggesting that predators exerted a strong pressure on aphid populations. Indeed, we found that predator abundances were greatest on plants under high AMF availability, following AMF-mediated increases in aphid abundance (Fig. 5.3). Greater predator abundances under high AMF availability may have counteracted the AMF-mediated increases in aphid population growth that we found previously (Meier and Hunter 2018). Similar to our findings, natural enemy abundances are greater on

mycorrhizal *Nicotiana rustica* (Wooley and Paine 2011) and *Phaseolus vulgaris* plants (Hoffmann et al. 2011b), and increases in predator abundance can compensate for AMF-mediated increases in herbivore performance (Hoffmann et al. 2011c).

While total predator abundances were greater on high AMF plants across milkweed species, the effects of AMF availability on the presence of spiders varied among milkweed species (Fig. 5.4) and were unrelated to aphid density. Colonization by spiders is influenced by both plant architecture (Robinson 1981, Rypstra 1983, Romero and Vasconcellos-Neto 2005) and volatile emissions (Nelson et al. 2012). Although we found no effect of AMF on plant height, AMF may have altered plant architecture, such as branching patterns, affecting colonization by spiders. In addition, AMF-induced changes in volatile emissions can alter natural enemy attraction, even in the absence of herbivores (Guerrieri et al. 2004). AMF-mediated changes in VOC emissions among milkweed species may have led to species-specific patterns of spider colonization.

In contrast to spiders, aphid midge fly oviposition and predation correlated strongly with aphid density, but the slopes of those relationships varied markedly among AMF treatments. Aphid midge flies laid more eggs, relative to aphid density, on *A. curassavica* plants without AMF than on plants under high AMF availability (Fig. 5.5a). Our findings contrast with those reported for aphid parasitoids (Hempel et al. 2009, Bennett et al. 2016) and predatory mites (Hoffmann et al. 2011c); parasitoids parasitize more aphids, and mites lay more eggs, on mycorrhizal plants. However, our findings are consistent with Gange (2003), who found rates of parasitism of leaf miners (*Diglyphus isaea*) to be lower on oxeye daisy (*Leucanthemum vulgare*) plants colonized by more AMF. Gange (2003) attributed this decrease in proportion parasitism to reduced parasitoid searching efficiency resulting from AMF-mediated increases in plant size. However, in our study, AMF did not affect plant height, and effects on plant biomass were transient. Instead, the reduced attraction of aphid midge flies may be due to AMF-induced changes in aphid and honeydew volatiles. Aphid midge flies are attracted to volatiles in aphid honeydew, not herbivore-induced plant volatiles (Watanabe et al. 2016), and AMF can alter the attractiveness of herbivores and their feces to predators (Hoffmann et al. 2011c). AMF, by

altering aphid honeydew volatiles, may lead aphids feeding on *A. curassavica* to be less attractive to aphid midge flies than are aphids feeding on plants without AMF.

Interestingly, the effects of AMF on midge fly oviposition appear decoupled from the subsequent effects of AMF on the abundance of midge fly larvae and the mortality that they impose on aphids. First, while aphid midge flies laid more eggs relative to aphid density on *A. curassavica* plants without AMF than on plants under high AMF availability, AMF did not affect the subsequent abundances of midge larvae. This may be due to increased midge survival when feeding on aphids from AMF-colonized plants; natural enemy performance is often improved when they are fed herbivores from AMF-colonized plants (Hempel et al. 2009, Hoffmann et al. 2011a, Bennett et al. 2016). The lack of effect of AMF on midge larva abundance may also result from effects of AMF on intraguild predation. Midge larvae are often consumed by coccinelids and lacewings (Lucas et al. 1998), predators that we commonly observed concurrent with aphid midge larvae on experimental plants. Effects of AMF availability on the attraction and abundances of these other predators may have resulted in the differences in midge larva densities compared to midge egg densities. However, due to low counts of coccinelids and lacewings overall, we were unable to evaluate this.

Second, midge larvae actually killed more aphids, relative to aphid density, on *A. curassavica* plants under high AMF availability than under medium AMF availability (Fig. 5.5b), even though midge larva abundances were unaffected by AMF availability. These results are consistent with previous studies that have reported higher rates of parasitism of aphids reared on mycorrhizal than non-mycorrhizal host plants, although the extent of increase in parasitism depends on AMF and plant identity (Gange et al. 2003, Hempel et al. 2009, Bennett et al. 2016). The different rates of aphid mortality that we observed among AMF treatments may have resulted from differences in prey quality for midge flies. *Aphis nerii* sequester cardenolides from their host plants, which provide effective resistance to natural enemies (Malcolm 1992). Concentrations of cardenolides sequestered by aphids are tightly linked with cardenolide concentrations in their host plants (Malcolm 1990). By altering plant cardenolide concentrations, AMF alter *A. nerii* sequestration of cardenolides (Meier and Hunter 2018). The greater cardenolide concentrations of *A. curassavica* plants under medium than high AMF availability at

the end of the season (Table 5.4) indicate that aphids may have been most toxic when feeding upon plants under medium AMF availability and least toxic on plants under high AMF availability. These differences in aphid sequestration could have driven the decreased rates of predation by midges on plants under high than medium AMF availability.

Overall, we found that the availability of AMF in soils had pervasive effects on herbivore-predator interactions in the field, indicating that AMF may help shape multitrophic interactions aboveground in natural systems. Because the availability of AMF inoculum, measured as infectivity and spore abundances, varies on small scales, (Carvalho et al. 2003, Wolfe et al. 2007), plants within a single population may experience substantial variation in AMF availability in soils. Based on our results, this variation in AMF abundance can result in differential attraction and abundance of herbivores and their predators, generating sufficient spatial variation in trophic interactions to affect large scale population dynamics (Riolo et al. 2015). Future studies should consider how natural AMF abundances influence the strength of top down and bottom up forces on herbivores to further elucidate the mechanisms by which AMF shape herbivore-predator dynamics in natural systems.

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Table 5.1. Effects of the availability of arbuscular mycorrhizal fungi (AMF) inoculum on plant traits in six milkweed species early in the growing season (June: 6-week old plants). Plant traits include the proportion of roots colonized by any AMF structures, number of leaves, plant height (cm), aboveground biomass (mg), belowground biomass (mg), proportion foliar water content, natural log-transformed latex exudation (mg), carbon concentration (%), nitrogen concentration (%), C/N ratio, phosphorous concentration (%), natural log-transformed cardenolide concentration (mg/g), cardenolide diversity, and cardenolide polarity. Numbers represent F-values and P-values from linear mixed models. Sample sizes per AMF x plant species combination for each trait are presented in Table 5.2.

	AMF		**Plant species**		**AMF x Plant species**	
	F	P	F	P	F	P
Proportion of roots colonized by AMF	$F_{2,161}$=60.72	<.0001***	$F_{5,161}$=1.95	0.0895*	$F_{10,161}$=1.97	0.0394**
Number of Leaves	$F_{2,161}$=0.25	0.7754	$F_{5,161}$=53.22	<.0001***	$F_{10,161}$=0.88	0.5498
Height	$F_{2,161}$=3.51	0.0322**	$F_{5,161}$=52.52	<.0001***	$F_{10,161}$=0.66	0.7628
Aboveground biomass	$F_{2,161}$=2.21	0.113	$F_{5,161}$=60.77	<.0001***	$F_{10,161}$=2.33	0.0137**
Belowground biomass	$F_{2,160}$=3.45	0.034**	$F_{5,160}$=29.98	<.0001***	$F_{10,160}$=1.63	0.1037
Foliar water content	$F_{2,161}$=6.42	0.0021**	$F_{5,161}$=45.64	<.0001***	$F_{10,161}$=1.13	0.342
Latex exudation	$F_{2,161}$=1.4	0.2503	$F_{5,161}$=58.18	<.0001***	$F_{10,161}$=1.01	0.4365
Carbon concentration	$F_{2,126}$=1.9	0.1543	$F_{5,126}$=17.77	<.0001***	$F_{10,126}$=1.9	0.0513*
Nitrogen concentration	$F_{2,126}$=0.66	0.5203	$F_{5,126}$=9.09	<.0001***	$F_{10,126}$=0.93	0.5105*
C/N ratio	$F_{2,126}$=0.61	0.5473	$F_{5,126}$=11.87	<.0001***	$F_{10,126}$=1.28	0.2486
Phosphorous concentration	$F_{2,159}$=0.59	0.554	$F_{5,159}$=23.06	<.0001***	$F_{10,159}$=0.85	0.5792
Cardenolide concentration	$F_{2,134}$=0.29	0.7509	$F_{4,134}$=202.34	<.0001***	$F_{8,134}$=0.74	0.6536
Cardenolide diversity	$F_{2,112}$=3.91	0.0228**	$F_{4,112}$=98.56	<.0001***	$F_{8,112}$=1.82	0.0803*
Cardenolide polarity	$F_{2,112}$=0.4	0.6739	$F_{4,112}$=25.97	<.0001***	$F_{8,112}$=1.41	0.2005

***P < 0.001, **P < 0.05, *P < 0.1.

Table 5.2. The mean amounts ± standard error and sample size (in parentheses) of plant traits early in the growing season (June: 6-week old plants) under zero, medium, and high levels of AMF inoculum availability. Plant traits include the proportion of roots colonized by AMF structures, number of leaves, plant height (cm), latex exudation (mg), aboveground biomass (mg), belowground biomass (mg), proportion foliar water content, carbon concentration (%), nitrogen concentration (%), C/N ratio, phosphorous concentration (%), cardenolide concentration (mg/g), cardenolide diversity, and cardenolide polarity.

Plant species	AMF	Proportion AMF colonization	Number of leaves	Plant height	Latex exudation	Aboveground biomass	Belowground biomass
A. curassavica	Zero	0 ± 0 (10)	12.1 ± 0.4 (10)	9.7 ± 0.4 (10)	0.3 ± 0.03 (10)	306.2 ± 27.8 (10)	212.2 ± 21.8 (10)
	Medium	0.02 ± 0.01 (10)	11.4 ± 0.6 (10)	9.8 ± 0.3 (10)	0.3 ± 0.04 (10)	297.2 ± 11.9 (10)	227 ± 14.2 (10)
	High	0.21 ± 0.05 (10)	14 ± 0.6 (10)	11.3 ± 0.6 (10)	0.3 ± 0.03 (10)	449.1 ± 30.7 (10)	310.4 ± 26 (10)
A. incarnata	Zero	0 ± 0 (10)	21.6 ± 1.7 (10)	25.8 ± 2.1 (10)	0.2 ± 0.03 (10)	505.5 ± 35.6 (10)	544.2 ± 57 (10)
	Medium	0.07 ± 0.03 (10)	19.1 ± 1.6 (10)	25 ± 2 (10)	0.2 ± 0.04 (10)	454.6 ± 46.9 (10)	518 ± 58 (10)
	High	0.13 ± 0.03 (10)	20.9 ± 1.8 (10)	30.8 ± 3.2 (10)	0.3 ± 0.03 (10)	514.1 ± 36 (10)	575.3 ± 57.2 (10)
A. speciosa	Zero	0 ± 0 (10)	15.4 ± 1 (10)	12.6 ± 0.8 (10)	1.4 ± 0.34 (10)	190.2 ± 23.7 (10)	212.8 ± 34.3 (10)
	Medium	0.14 ± 0.04 (10)	17.8 ± 0.8 (10)	14 ± 1.3 (10)	1.9 ± 0.38 (10)	310.6 ± 53.9 (10)	412.1 ± 97 (10)
	High	0.15 ± 0.03 (10)	14.9 ± 1.2 (10)	15 ± 2.6 (10)	1.2 ± 0.27 (10)	238.3 ± 42.4 (10)	307.3 ± 75.8 (10)
A. sullivantii	Zero	0 ± 0 (10)	13.3 ± 0.8 (10)	15.5 ± 0.9 (10)	0.7 ± 0.13 (10)	144.9 ± 24.9 (10)	175.1 ± 38.2 (10)
	Medium	0.15 ± 0.05 (10)	11.7 ± 0.7 (10)	13.7 ± 1 (10)	0.8 ± 0.09 (10)	147.6 ± 23.3 (10)	184.2 ± 36.9 (10)
	High	0.21 ± 0.04 (10)	12.4 ± 0.9 (10)	16 ± 1.5 (10)	0.7 ± 0.14 (10)	153.5 ± 21.7 (10)	194.3 ± 27.4 (10)
A. syriaca	Zero	0 ± 0 (10)	12.8 ± 0.7 (10)	11.1 ± 0.5 (10)	0.8 ± 0.11 (10)	267.4 ± 21.4 (10)	303.4 ± 32.4 (10)
	Medium	0.14 ± 0.05 (10)	13 ± 0.5 (10)	10.5 ± 0.3 (10)	1 ± 0.13 (10)	314.1 ± 23.2 (10)	498.2 ± 57.7 (10)
	High	0.25 ± 0.04 (10)	11.7 ± 0.8 (10)	10.6 ± 0.6 (10)	1 ± 0.14 (10)	273.5 ± 18.9 (10)	319.3 ± 13.5 (9)
A. verticillata	Zero	0 ± 0 (10)	27.2 ± 3.3 (10)	18.3 ± 1.6 (10)	0.2 ± 0.02 (10)	123.8 ± 31 (10)	109.1 ± 38.2 (10)
	Medium	0.08 ± 0.02 (10)	28.6 ± 2.5 (10)	16.9 ± 1 (10)	0.2 ± 0.03 (10)	113.6 ± 14.3 (10)	131.6 ± 27.6 (10)
	High	0.29 ± 0.05 (10)	31.4 ± 3.5 (10)	19.4 ± 1.8 (10)	0.2 ± 0.02 (10)	129.6 ± 22.5 (10)	150.4 ± 40.9 (10)

Table 5.2, continued.

Foliar water content	Carbon concentration	Nitrogen concentration	C/N ratio	Phosphorous concentration	Cardenolide concentration	Cardenolide diversity	Cardenolide polarity
0.59 ± 0.04 (10)	42.11 ± 0.14 (10)	2.02 ± 0.13 (10)	21.6 ± 1.4 (10)	0.24 ± 0.02 (10)	5.03 ± 0.65 (10)	2.09 ± 0.07 (10)	0.54 ± 0.01 (10)
0.64 ± 0.03 (10)	42.01 ± 0.12 (10)	1.96 ± 0.12 (10)	22.11 ± 1.3 (10)	0.23 ± 0.02 (10)	6.15 ± 0.82 (10)	2.07 ± 0.07 (10)	0.56 ± 0.02 (10)
0.54 ± 0.03 (10)	41.83 ± 0.07 (10)	2.03 ± 0.12 (10)	21.21 ± 1.2 (10)	0.26 ± 0.01 (10)	5.49 ± 0.76 (10)	2.02 ± 0.07 (10)	0.54 ± 0.02 (10)
0.57 ± 0.03 (10)	42.34 ± 0.15 (10)	1.82 ± 0.08 (10)	23.69 ± 1 (10)	0.28 ± 0.02 (10)	0 ± 0 (10)	NA	NA
0.64 ± 0.01 (10)	42.2 ± 0.23 (10)	1.81 ± 0.1 (10)	23.85 ± 1.1 (10)	0.31 ± 0.02 (10)	0 ± 0 (10)	NA	NA
0.55 ± 0.03 (10)	42.5 ± 0.16 (10)	1.84 ± 0.13 (10)	24.34 ± 2 (10)	0.31 ± 0.03 (10)	0 ± 0 (10)	NA	NA
0.73 ± 0.03 (10)	40.21 ± 0.34 (8)	2.54 ± 0.12 (8)	16.04 ± 0.7 (8)	0.24 ± 0.01 (10)	0.1 ± 0.03 (10)	0.43 ± 0.17 (9)	0.47 ± 0.07 (9)
0.77 ± 0.01 (10)	40.96 ± 0.22 (10)	2.36 ± 0.18 (10)	18.3 ± 1.5 (10)	0.25 ± 0.01 (10)	0.14 ± 0.07 (10)	0.55 ± 0.16 (9)	0.43 ± 0.06 (9)
0.79 ± 0.01 (10)	39.67 ± 0.67 (8)	2.5 ± 0.13 (8)	16.15 ± 0.9 (8)	0.27 ± 0.02 (10)	0.16 ± 0.05 (10)	0.82 ± 0.13 (10)	0.33 ± 0.03 (10)
0.68 ± 0.01 (10)	40.54 ± 0.45 (6)	2.09 ± 0.15 (6)	19.84 ± 1.3 (6)	0.22 ± 0.01 (10)	1.63 ± 0.42 (10)	0.83 ± 0.12 (9)	0.16 ± 0.01 (9)
0.72 ± 0.02 (10)	39.89 ± 0.82 (7)	2.15 ± 0.05 (7)	18.53 ± 0.3 (7)	0.24 ± 0.01 (10)	1.51 ± 0.32 (10)	0.87 ± 0.11 (10)	0.16 ± 0.01 (10)
0.7 ± 0.03 (10)	39.48 ± 0.75 (8)	2.32 ± 0.14 (8)	17.37 ± 1 (8)	0.24 ± 0.02 (10)	1.04 ± 0.23 (10)	1.12 ± 0.12 (8)	0.2 ± 0.02 (8)
0.51 ± 0.05 (10)	41.7 ± 0.22 (10)	2.39 ± 0.09 (10)	17.66 ± 0.6 (10)	0.26 ± 0.02 (10)	0.52 ± 0.11 (10)	0.86 ± 0.12 (10)	0.5 ± 0.03 (10)
0.56 ± 0.03 (10)	41.24 ± 0.2 (10)	1.93 ± 0.11 (10)	22.08 ± 1.3 (10)	0.23 ± 0.01 (10)	0.27 ± 0.05 (10)	0.34 ± 0.14 (10)	0.48 ± 0.07 (10)
0.46 ± 0.05 (10)	41.85 ± 0.21 (10)	2.22 ± 0.1 (10)	19.18 ± 0.8 (10)	0.27 ± 0.01 (10)	0.38 ± 0.07 (10)	0.66 ± 0.09 (10)	0.47 ± 0.05 (10)
0.75 ± 0.01 (10)	42.27 ± 0.09 (2)	2.33 ± 0.08 (2)	18.2 ± 0.6 (2)	0.4 ± 0.02 (9)	0.02 ± 0.01 (10)	0.25 ± 0.25 (4)	0.32 ± 0.15 (4)
0.77 ± 0.01 (10)	39.95 ± 1.75 (4)	2.37 ± 0.29 (4)	17.43 ± 1.6 (4)	0.4 ± 0.05 (9)	0.02 ± 0.01 (10)	0 ± 0 (5)	0.5 ± 0.15 (5)
0.75 ± 0.01 (10)	42.48 ± 0.31 (2)	1.92 ± 0.57 (2)	24.22 ± 7 (2)	0.36 ± 0.03 (10)	0.01 ± 0.01 (10)	0.33 ± 0.19 (4)	0.56 ± 0.11 (4)

Table 5.3. Effects of the availability of AMF inoculum on plant traits in six milkweed species at the end of the growing season (October: 5-month old plants). Plant traits include the proportion of roots colonized by AMF structures, average plant height from August 13 to October 1, 2015, aboveground biomass (mg), belowground biomass (mg), natural log-transformed foliar cardenolide concentration (mg/g), cardenolide diversity, and cardenolide polarity. Numbers represent F-values and P-values from linear mixed models. Sample sizes for each plant trait per AMF x plant species combination are presented in Table 5.4.

	AMF		**Plant species**		**AMF x Plant species**	
	F	**P**	**F**	**P**	**F**	**P**
Proportion of roots colonized by AMF	$F_{2,319}$=422.37	<.0001***	$F_{5,319}$=1.82	0.1080	$F_{10,319}$=1.50	0.1388
Average plant height	$F_{2,320}$=0.42	0.6575	$F_{5,320}$=347.93	<.0001***	$F_{10,320}$=0.20	0.9965
Aboveground biomass	$F_{2,204}$=0.29	0.752	$F_{5,204}$=318.4	<.0001***	$F_{10,204}$=0.42	0.9375
Belowground biomass	$F_{2,318}$=0.32	0.7276	$F_{5,318}$=216.26	<.0001***	$F_{10,318}$=1.64	0.0939*
Foliar cardenolide concentration	$F_{2,179}$=3.3	0.039**	$F_{3,179}$=309.24	<.0001***	$F_{6,179}$=2.52	0.0229**
Cardenolide diversity	$F_{2,151}$=5.77	0.0038**	$F_{3,151}$=492.18	<.0001***	$F_{6,151}$=1.46	0.1953
Cardenolide polarity	$F_{2,151}$=4.04	0.0195**	$F_{3,151}$=467.12	<.0001***	$F_{6,151}$=1.09	0.37

***P < 0.001, **P < 0.05, *P < 0.1

Table 5.4. The mean amounts ± standard error and sample size (in parentheses) of plant traits at the end of the growing season (October: 5-month old plants), under zero, medium, and high levels of AMF inoculum availability. Plant traits include the proportion of roots colonized by any AMF structures, average plant height from August 13 to October 1, 2015, aboveground biomass (mg), belowground biomass (mg), cardenolide concentration (mg/g), cardenolide diversity, and cardenolide polarity.

Plant species	AMF	Proportion AMF colonization	Plant height	Aboveground biomass	Belowground biomass	Cardenolide concentration	Cardenolide diversity	Cardenolide polarity
A. curassavica	Zero	0.01 ± 0.01 (20)	32.4 ± 1.1 (20)	1905.0 ± 87.0 (20)	1829.2 ± 91.5 (20)	2.65 ± 0.25 (20)	2.44 ± 0.04 (20)	0.62 ± 0.01 (20)
	Medium	0.48 ± 0.04 (20)	31.4 ± 1 (20)	1968.8 ± 73.0 (20)	2051.6 ± 48.3 (20)	3.13 ± 0.31 (20)	2.41 ± 0.05 (20)	0.61 ± 0.01 (20)
	High	0.53 ± 0.02 (15)	32.1 ± 1.5 (15)	1931.4 ± 95.8 (15)	1901.1 ± 116.8 (15)	2.31 ± 0.39 (15)	2.23 ± 0.15 (15)	0.55 ± 0.04 (15)
A. incarnata	Zero	0 ± 0 (21)	57.4 ± 3.3 (21)	1299.7 ± 77.6 (21)	4483.7 ± 152.9 (21)	0.15 ± 0.13 (17)	0.15 ± 0.12 (15)	0.14 ± 0.02 (15)
	Medium	0.47 ± 0.05 (19)	54.2 ± 2.8 (19)	1226.5 ± 52.6 (19)	4033.3 ± 145.2 (19)	0.02 ± 0 (18)	0.04 ± 0.04 (12)	0.15 ± 0.04 (12)
	High	0.52 ± 0.03 (20)	56.4 ± 2.8 (20)	1353.6 ± 64.3 (20)	4321.2 ± 145.9 (19)	0.03 ± 0.01 (19)	0.05 ± 0.05 (12)	0.12 ± 0.01 (12)
A. speciosa	Zero	0 ± 0 (20)	2.6 ± 0.5 (20)	72.6 ± 19.9 (8)	968.4 ± 92.7 (20)	0.42 ± 0.16 (8)	0.66 ± 0.25 (7)	0.2 ± 0.03 (7)
	Medium	0.45 ± 0.05 (19)	3 ± 0.8 (20)	184.2 ± 62.0 (10)	1223.7 ± 141.2 (20)	0.12 ± 0.04 (9)	0.15 ± 0.12 (6)	0.14 ± 0.01 (6)
	High	0.52 ± 0.04 (20)	3.4 ± 0.7 (20)	104.9 ± 34.0 (9)	1167.5 ± 137.3 (20)	0.07 ± 0.04 (9)	0.09 ± 0.09 (7)	0.13 ± 0.01 (7)
A. sullivantii	Zero	0 ± 0 (15)	11.5 ± 1.8 (15)	287.9 ± 89.6 (2)	1148.6 ± 100.0(15)	NA	NA	NA
	Medium	0.39 ± 0.05 (16)	12.5 ± 1.6 (16)	295.2 ± 150.6 (3)	1292.6 ± 155.7 (16)	2.21 ± 1.06 (3)	0.7 ± 0.35 (3)	0.13 ± 0.01 (3)
	High	0.42 ± 0.04 (15)	13.8 ± 1.4 (15)	351.8 ± 101.0 (2)	1566.5 ± 138.6 (15)	1.75 ± 0.89 (2)	0.77 ± 0.05 (2)	0.13 ± 0 (2)
A. syriaca	Zero	0 ± 0 (19)	7.8 ± 1.5 (19)	176.0 ± 40.4 (3)	2369.5 ± 141.1 (18)	NA	NA	NA
	Medium	0.4 ± 0.05 (21)	8.2 ± 1.1 (21)	183.2 ± 34.8 (8)	2363.4 ± 152.1 (21)	0.15 ± 0.12 (4)	0.49 ± 0.25 (3)	0.34 ± 0.16 (3)
	High	0.6 ± 0.02 (20)	8.3 ± 1 (20)	196.6 ± 67.8 (6)	2111.3 ± 153.7 (20)	0.21 ± 0.11 (3)	0.26 ± 0.26 (3)	0.12 ± 0.01 (3)
A. verticillata	Zero	0 ± 0 (20)	35.7 ± 2.5 (20)	358.6 ± 25.1 (19)	2287.4 ± 143.2 (20)	0.18 ± 0.03 (19)	0.2 ± 0.08 (15)	0.12 ± 0.01 (15)
	Medium	0.39 ± 0.05 (18)	34.4 ± 2.9 (18)	459.4 ± 42.3 (18)	2401.3 ± 175.6 (18)	0.14 ± 0.02 (18)	0.2 ± 0.08 (17)	0.12 ± 0.01 (17)
	High	0.5 ± 0.03 (22)	35.8 ± 1.8 (22)	420.5 ± 25.4 (22)	2375.8 ± 107.6 (22)	0.2 ± 0.03 (22)	0.15 ± 0.06 (20)	0.12 ± 0.01 (20)

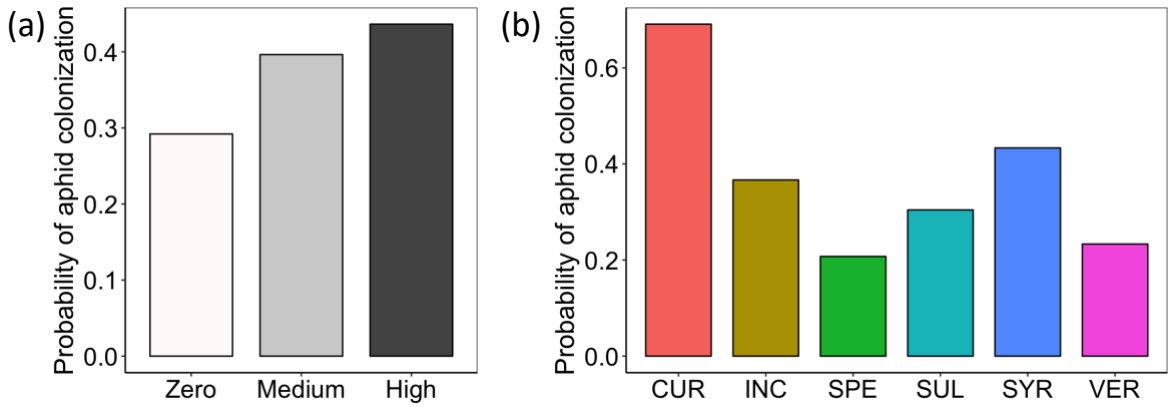

Figure 5.1. Effects of a) AMF availability and b) milkweed species on the probability of aphid colonization during the growing season. Sample sizes are 109-111 plants per AMF treatment and 46-60 plants per plant species. The abbreviations for each plant species are: CUR = *A. curassavica*, INC = *A. incarnata*, SPE = *A. speciosa*, SUL = *A. sullivantii*, SYR = *A. syriaca*, VER = *A. verticillata.*

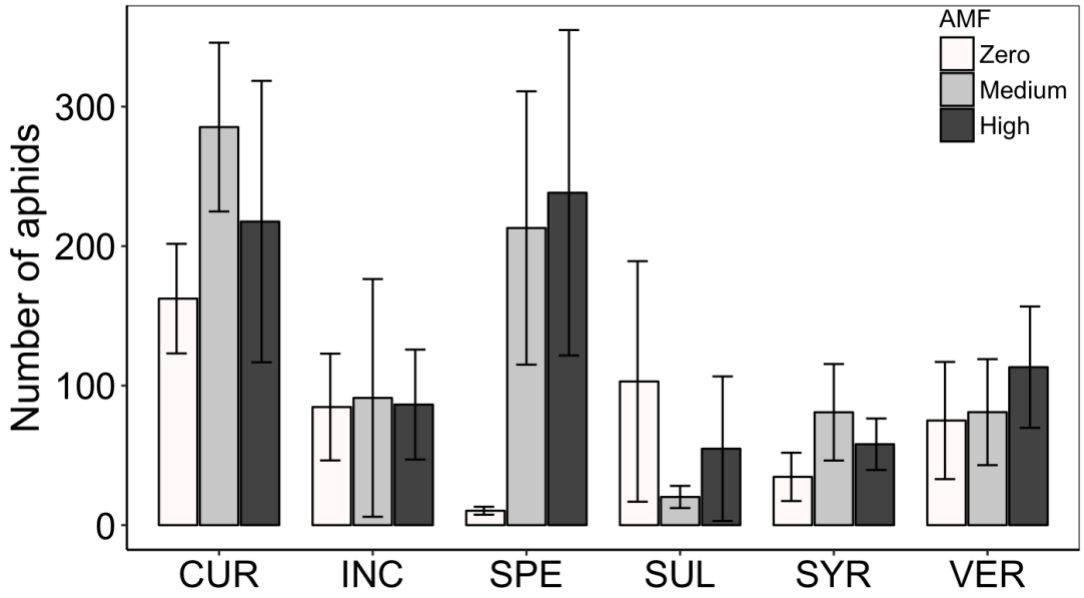

Figure 5.2. Effects of AMF availability on the cumulative number of aphids present on milkweed plants. Due to low rates of aphid colonization, sample sizes range from 2-15 plants per plant species x AMF treatment, with a median of 5 plants per treatment. The abbreviations for each plant species are: CUR = *A. curassavica*, INC = *A. incarnata*, SPE = *A. speciosa*, SUL = *A. sullivantii*, SYR = *A. syriaca*, VER = *A. verticillata*.

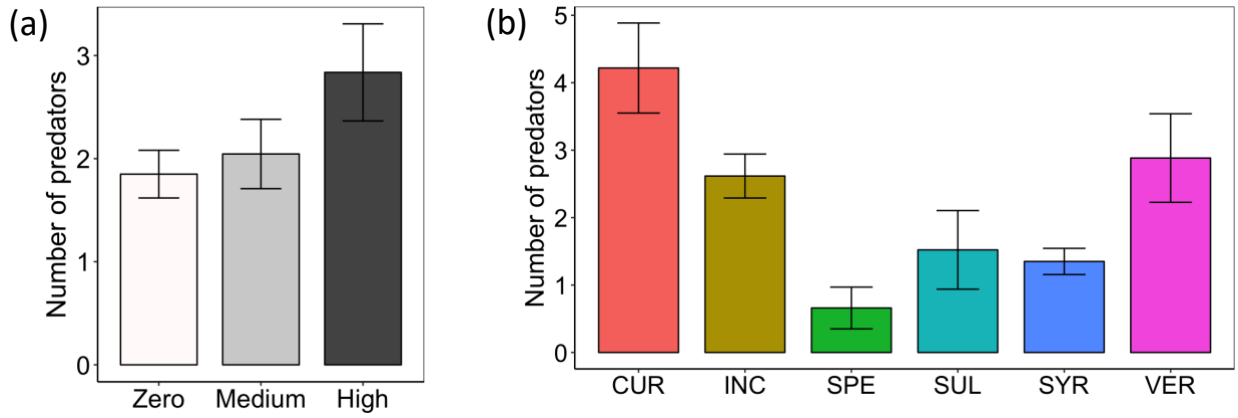

Figure 5.3. Effects of a) AMF availability and b) milkweed species on the cumulative number of predators on plants over the growing season. Sample sizes range from 109-111 plants per AMF treatments and 46-60 plants per plant species. Bars represent the mean ± 1 SE. The abbreviations for each plant species are: CUR = *A. curassavica*, INC = *A. incarnata*, SPE = *A. speciosa*, SUL = *A. sullivantii*, SYR = *A. syriaca*, VER = *A. verticillata*. Note that the average number of predators per treatment appears small because many plants hosted zero predators. While averages are presented for ease of visualization, data were analyzed using a negative binomial distribution to account for substantial clumping of predators on plants.

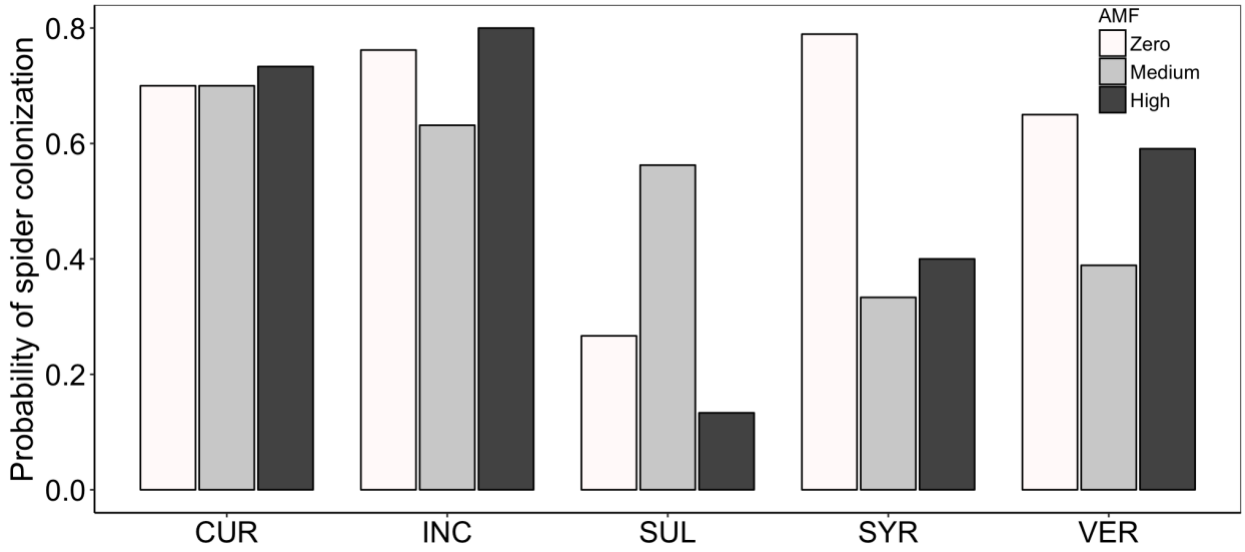

Figure 5.4. Effects of AMF availability on the probability of spider colonization during the growing season among milkweed species. Because no spiders colonized *A. speciosa* plants under zero or medium AMF availability, *A. speciosa* plants were excluded. Sample sizes are 15-22 plants per treatment combination. The abbreviations for each plant species are: CUR = *A. curassavica,* INC = *A. incarnata,* SUL = *A. sullivantii,* SYR = *A. syriaca,* VER = *A. verticillata.*

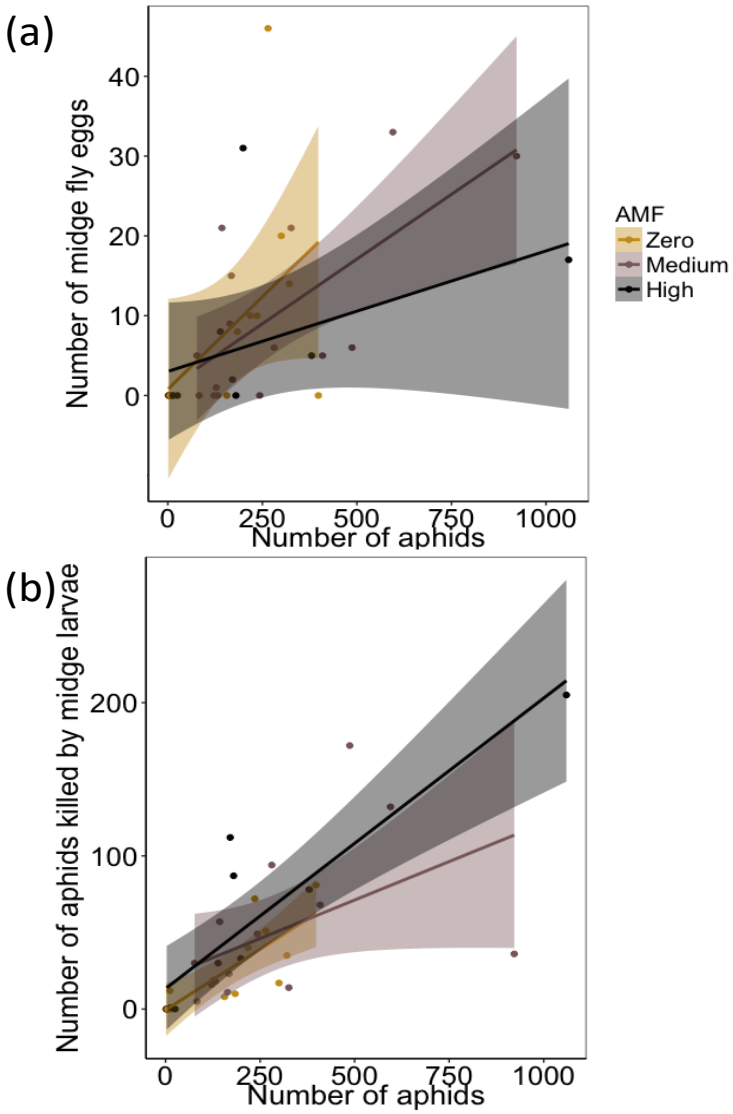

Figure 5.5. a) Effects of AMF availability on the cumulative number of midge (*Aphidoletes aphidimyza*) eggs laid on *A. curassavica* plants relative to the cumulative number of aphids. b) Effects of AMF availability on the cumulative number of aphids killed by midge larvae over the growing season relative to the number of live aphids present on *A. curassavica* plants. Each point represents cumulative values for one plant. Sample sizes range from 10-15 plants per AMF treatment.

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Chapter VI
Conclusion

Multitrophic interactions are shaped by both top-down and bottom up forces. Mutualisms, by altering partner phenotype, may directly and indirectly alter the strength of these forces, influencing community structure and population dynamics. In terrestrial ecosystems, both top-down and bottom-up forces travel with ease across the traditional soil "boundary", with plants connecting the interactions that occur between above and belowground organisms (van der Putten et al. 2001, van Dam and Heil 2011). As a result, mutualists belowground impact the performance of aboveground insect herbivores (Erb et al. 2008, Koricheva et al. 2009, Pineda et al. 2010, Rasmann et al. 2017) and the resistance of herbivores to their natural enemies from the top down (Gange et al. 2003, Rasmann and Turlings 2007, Rasmann et al. 2017, Tao et al. 2017). Similarly, herbivores feeding on leaf tissue alter the performance of mutualists belowground (Gehring and Bennett 2009, Barto and Rillig 2010). Although ubiquitous, the mechanisms and ecological consequences of such interactions are just beginning to be understood (Erb et al. 2009, Papadopoulou and van Dam 2017, De Deyn 2017).

In my dissertation, I combined a series of manipulative experiments to evaluate how arbuscular mycorrhizal fungi (AMF) influence multitrophic interactions. First, I explored how arbuscular mycorrhizal fungi mediate herbivore-induction of plant defenses above and belowground, and how herbivores influence AMF colonization of roots. Second, I assessed how AMF-mediated changes in plant nutritive and defensive traits affect toxin sequestration and performance of two specialist herbivores. Third, I examined the effects of AMF on constitutive and herbivore-induced volatile emissions. Lastly, I evaluated the ecological relevance of AMF on multitrophic interactions in the field. Below, I summarize the results from these four chapters.

Chapter II: Arbuscular mycorrhizal fungi mediate herbivore-induction of plant defenses differently above and belowground

By altering plant phenotype, organisms above and belowground can impact one another substantially (Van der Putten et al. 2001, Bezemer and van Dam 2005, Erb et al. 2008, Pineda et al. 2010, van Dam and Heil 2011, Johnson et al. 2012, Stam et al. 2014). In this chapter, I evaluated the impacts of AMF on constitutive and herbivore-induced plant defenses simultaneously in above and belowground plant tissues. We demonstrated that milkweeds colonized by AMF generally produce tougher and more toxic leaves than do AMF-free plants. Furthermore, the relative induction or suppression of foliar defenses by aphids and caterpillars is altered by the availability of AMF inoculum. In contrast, AMF inoculum availability induces plant species-specific changes in the chemical defenses of roots, but does not influence the suppression (by aphids) or induction (by caterpillars) of root chemical defenses. Finally, herbivore feeding leads to substantial changes in levels of AMF colonization of roots after just a few days, with the magnitude and direction of those changes varying with plant and herbivore species. This last result is important because it completes a fundamental feedback loop from populations of microbial root mutualists belowground, through alterations in plant phenotype aboveground, to changes in the effects of herbivore feeding on plant traits, and back down to the root mutualists (Hunter 2016). Our findings suggest that indirect interactions between AMF and herbivores may have community-wide consequences by altering plant phenotype both above and belowground.

Chapter III. Mycorrhizae alter toxin sequestration and performance of two specialist herbivores

By altering plant nutritive and defensive traits, as we demonstrated in Chapter II, AMF may influence the both herbivore performance (Hartley and Gange 2009, Koricheva et al. 2009) and resistance to their natural enemies. In this chapter, we documented the impacts of AMF on toxin sequestration by specialist herbivores, while measuring simultaneously effects on herbivore performance. We demonstrated that aphids and caterpillars sequester higher concentrations of cardenolides from plants inoculated with AMF, following AMF-mediated increases in foliar cardenolide concentrations. In addition, AMF availability influences the performance of both aphids and caterpillars on milkweed, though in different ways. On all milkweed species, aphid

performance varies nonlinearly with increasing AMF inoculum availability, with lowest performance under medium levels of inoculum availability and highest performance under high inoculum availability. In contrast, while caterpillar survival varies markedly with AMF inoculum availability, it does so in a plant species-specific manner, and caterpillar growth is unaffected by AMF. Aphid performance declined with increasing foliar cardenolide concentrations, while caterpillar survival increased with aboveground biomass. Our findings suggest that by altering plant phenotype, the availability of AMF in soil has the potential to influence both the top-down (via sequestration) and the bottom up (via plant defense and nutrition) forces that operate on milkweed herbivores.

Chapter IV. Mycorrhizae alter constitutive and herbivore-induced volatile emissions by milkweeds

AMF may shape multitrophic interactions not only by altering plant quality for herbivores (Chapters II and III) (Hartley and Gange 2009, Koricheva et al. 2009), but also by altering plant volatile emissions (Bruce et al. 2005, Bruce and Pickett 2011). Herbivores utilize plant volatile blends to identify appropriate host plants, and natural enemies use volatiles to locate their herbivore prey (Kessler and Baldwin 2001, Turlings and Erb 2018). By altering plant volatile emissions, AMF may alter the attraction of herbivores and their natural enemies to plants. In this chapter, I investigated how the amount of AMF available to plants influences constitutive and aphid-induced VOC emissions in two milkweed species (*A. incarnata* and *A. curassavica*). We found that high AMF availability increases emissions of total VOCs, green leafy volatiles, and methyl salicylate in *A. curassavica* but decreases emissions in *A. incarnata*. In contrast, aphids consistently increase emissions of 6-methyl-5-hepten-2-one and benzeneacetaldehyde in both *A. incarnata* and *A. curassavica*, and AMF does not affect these emissions. Importantly, aphids suppress emissions of individual terpenes in the absence of AMF. However, high AMF availability suppresses terpene emissions to levels equivalent to those mediated by aphids, such that aphid damage on plants under high AMF availability does not further suppress terpene emissions. Our findings suggest that by altering milkweed VOC profiles, AMF may generate subsequent effects on herbivore and natural enemy attraction, and that AMF may affect the indirect defenses of these milkweed species differently.

Chapter V. Arbuscular mycorrhizal fungi alter herbivore-predator interactions

In Chapters II-IV, I demonstrated that the availability of AMF in soil influences strongly plant quality for herbivores, toxin sequestration and performance of herbivores, and plant constitutive and herbivore-induced volatile emissions. These findings suggest that AMF may affect interactions among trophic levels by altering the strength of top-down and bottom-up forces acting on herbivore populations. In this chapter, I assessed how AMF influence the colonization of milkweed species by herbivores and their natural enemies in the field. We found that AMF increase the probability of aphid colonization consistently among plant species but, after colonization, alter subsequent aphid abundances differentially among plant species. Following AMF-mediated increases in aphid colonization and abundance, total predator abundances are greatest on plants under high AMF availability, consistently among plant species. Effects of AMF on individual predators are more complicated; the probability of spider colonization varies with AMF availability differentially among plant species, irrespective of aphid density. In contrast, aphid midge fly oviposition and predation of aphids on *A. curassavica* plants vary strongly with aphid density and the amount of AMF available to their host plants. Most notably, the per capita mortality rate imposed by midge flies on aphids varies with AMF availability. Our findings suggest that the availability of AMF in soils may have pervasive effects on herbivore-predator dynamics in the field.

Synthesis and future directions

This dissertation illustrates that mutualisms below ground influence multitrophic interactions strongly by altering the strength of the top-down and bottom-up forces that shape such interactions. Specifically, I show that AMF influence bottom-up forces by altering plant nutritive and defensive traits and constitutive volatile emissions (Chapter II-IV), affecting herbivore performance and survival (Chapter III). In addition, I demonstrate that AMF influence top-down forces by increasing herbivore resistance to their natural enemies (via toxin sequestration) (Chapter III) and altering herbivore-induced plant volatiles (Chapter IV). I show that the effects of AMF on these forces are ecologically relevant under field conditions; the availability of AMF influenced both herbivore and predator colonization and abundance (Chapter V). These results indicate that belowground mutualists of plants have pervasive effects on herbivore-predator interactions in the field.

In addition, I demonstrate that herbivores alter AMF colonization of roots. This last result is important because it completes a fundamental feedback loop from populations of microbial root mutualists belowground, through alterations in plant phenotype aboveground, to changes in the effects of herbivore feeding on plant traits, and back down to the root mutualists (Hunter 2016). By altering AMF colonization of roots, herbivores may affect the overall abundance of AMF in soil (Powell et al. 2009). Ultimately, this could feed back to affect plant defenses and the performance of herbivores. In addition, because AMF play a key role in ecosystem processes, such as global C cycling (Bago et al. 2000), P cycling (Jansa et al. 2011), and soil aggregation (Brito et al. 2008), the influence of herbivores on AMF could have ecosystem-level consequences. Future studies should consider how natural AMF abundances alter plant phenotypes, plant-herbivore-natural enemy interactions, and plant-soil feedbacks at individual, community, and ecosystem scales.

While this dissertation demonstrates that mutualists below ground shape multitrophic interactions, it remains difficult to anticipate the direction or magnitude of these effects. This is due to the diversity of mechanisms by which AMF can affect multitrophic interactions (above), and the highly context-dependent nature of the influence of AMF on each mechanism. For example, under greenhouse conditions in the absence of predators, aphid per capita growth rates and body mass increase nonlinearly with increasing AMF availability, consistently among plant species. Aphid performance was greatest under high AMF availability and least under medium AMF availability. Aphid sequestration of cardenolides, however, was greatest under medium AMF availability, and least on plants without AMF. Therefore, while aphids on host plants under medium AMF availability have the lowest performance, they may also be the best defended against their natural enemies. Indeed, rates of mortality of aphids by aphid midge flies varied with AMF availability, potentially due to differences in toxin sequestration by herbivores. In addition, while effects of AMF on aphid performance were consistent across milkweed species, AMF had species-specific effects on caterpillar survival. Therefore, the effects of AMF on herbivore populations will depend on the specific herbivore involved.

Our results suggest that AMF may drive herbivore abundances by affecting top-down pressure on herbivore populations in the field, counteracting the bottom-up effects of AMF on aphid population growth. In the absence of predators, aphid growth was greatest on plants under high AMF availability and least on plants under medium AMF availability. Yet, in the field, aphid abundances were either greatest on the same plant species under medium AMF availability or were unaffected by AMF. These differences in aphid abundance among studies are likely driven by AMF effects on predators. Total predator abundances were greatest on plants under high AMF availability, which may have counteracted the AMF-mediated increases in aphid populations under high AMF availability. To further elucidate the relative strength of AMF on top-down versus bottom-up forces in the field, future field experiments in which predators are both included and excluded (e.g. Mooney et al. 2010), are necessary to understand how AMF influence the strength of top-down versus bottom-up forces acting on herbivore populations.

Furthermore, while total predator abundances were greatest on plants under high AMF availability, the presence of specific predators, such as spiders, varied with AMF availability differentially among plant species. Therefore, AMF may influence the structure of predator communities. Indeed, we have preliminary evidence which suggests that predator communities vary with AMF availability (Meier and Hunter, in prep). As natural enemies, and particular communities of natural enemies, vary in their ability to suppress herbivore populations (Snyder and Ives 2001, 2003, Snyder and Wise 2001, Crowder et al. 2010, Gontijo et al. 2015), AMF-mediated changes in predator communities may be a yet untested mechanism by which AMF may shape herbivore populations in the field.

Because AMF affect both top-down and bottom-up forces operating on herbivore populations, the effects of AMF on multitrophic interactions will also depend on the particular ecological context. For example, Helms (2004) found that *Aphis nerii* populations experience density-dependent parasitism, but that their population growth remains unlimited by natural enemies in the field. In contrast, predators exerted strong pressure on herbivore populations in our study; aphid populations persisted for an average of only three weeks before being driven extinct. In systems in which predators exert a weaker pressure on herbivore populations, AMF effects on plant quality for herbivores may drive herbivore abundances. In contrast, in systems

like ours in which herbivore populations are constrained by predators, AMF effects on the attraction of natural enemies and rates of predation may drive herbivore dynamics in the field. Similarly, variation in the availability of nutrients in soils will likely alter the effects of AMF on multitrophic interactions; the outcome of symbioses between plants and AMF depend on resource availability, often being beneficial in poor nutrient environments and detrimental in high resource environments (e.g. Johnson 2010, Hoeksema et al. 2010). Future work should evaluate how AMF may differentially affect multitrophic interactions in varying ecological contexts.

In addition, the strong effects of AMF on aphid population growth and abundance, as well as on predator colonization, illustrate that AMF may influence herbivore population dynamics, not just individual performance. To evaluate the potential effects of AMF on the population dynamics of herbivores, future studies should determine the effects of AMF on density dependent processes. For instance, experimental manipulations of aphid density under different availabilities of AMF could provide specific parameters of population growth, such as carrying capacity, and the form and strength of density dependence (e.g. Zehnder and Hunter 2008). Such experimentally-derived parameters could then be used in population growth simulations to determine how the availability of AMF influences the population dynamics of insect herbivores (Underwood and Rausher 2000, 2002).

In this dissertation, I considered only variation in the availability of a single species of AMF, *Funneliformis mosseae.* However, plants are naturally colonized by a community of AMF species (e.g. Jansa et al. 2003) and there is spatial variation in AMF communities from landscape (Öpik et al. 2006, 2013, Hazard et al. 2013) to meter scales (Davison et al. 2012). Therefore, it is likely that different combinations of AMF species colonize plants within a single community. The particular structure of AMF communities influences nutrient exchange (Argüello et al. 2016), with consequences for plant phenotypes (Bennett and Bever 2007, Thonar et al. 2014), herbivore performance (Gange et al. 2005, Wooley and Paine 2007, Vannette and Hunter 2013), and predator attraction (Gange et al. 2003, Wooley and Paine 2011). Therefore, to further elucidate how mycorrhizae influence multitrophic interactions, experiments using the natural variation in AMF communities are necessary. Furthermore, in natural systems, plants are often

connected by common mycorrhizal networks, through which plants can warn their neighbors of attack by herbivores, leading to induction of plant defenses prior to herbivore damage (Song et al. 2010, Babikova et al. 2013). While I considered plants individually in the studies presented here, such networks may also drive multitrophic interactions and should be considered.

Overall, we found that common belowground mutualisms, such as AMF, have strong effects on multitrophic interactions in the field by affecting both the top-down and bottom-up forces that operate on herbivore populations. Understanding the multifaceted ways in which mutualists belowground influence aboveground species interactions is essential to ultimately predict the effects of mutualists belowground on multitrophic interactions. Being able to do so has far-reaching applications. For example, the ability to manipulate microbial communities to enhance natural pest control in agricultural systems holds much promise (Lakshmanan et al. 2014, Lapsansky et al. 2016). However, most attempts to date have been relatively unsuccessful due to different results in experimental than in real-world settings (Lapsansky et al. 2016, Hart et al. 2018). This dissertation provides a first step towards evaluating mechanisms by which AMF affect multitrophic interactions. However, much more work is needed to ultimately predict the effects of mutualists belowground on multitrophic interactions aboveground.

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Appendix A

Supplementary materials for Chapter II

Table A2.1. Mean final number of aphids ± 1SE present in each plant species x AMF treatment after six days of feeding. Samples sizes are 15 aphid populations (=replicate plants) per plant species x AMF treatment.

Plant species	AMF inoculum availability	Final number of aphids
A. curassavica	Zero	45.3 ± 3.3
	Medium	38.9 ± 4.3
	High	53.3 ± 4.4
A. incarnata	Zero	52.3 ± 4.5
	Medium	54.3 ± 4.8
	High	72.3 ± 6.3
A. latifolia	Zero	39.0 ± 4.5
	Medium	31.1 ± 3.5
	High	46.5 ± 4.3
A. syriaca	Zero	40.7 ± 4.7
	Medium	39.6 ± 5.3
	High	61.0 ± 7.2

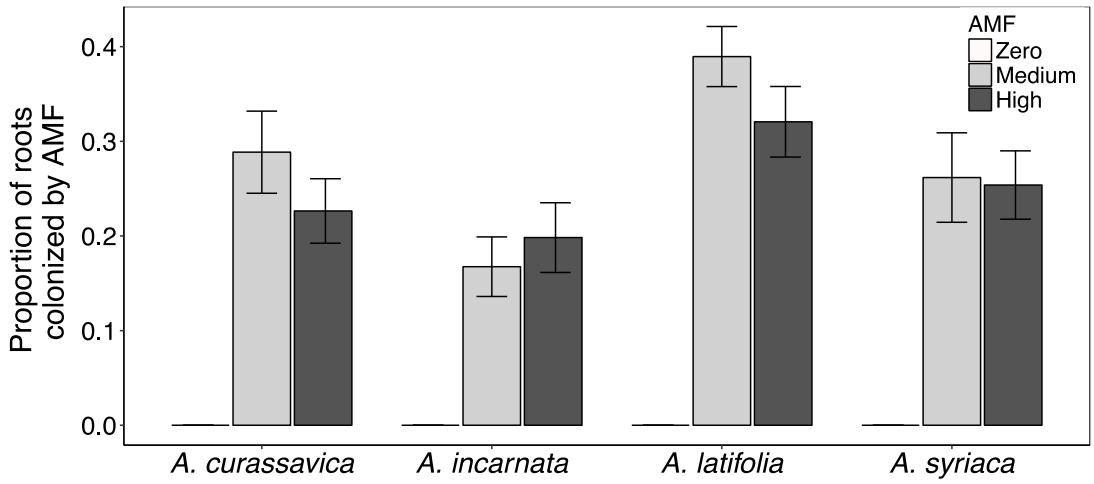

Figure A2.1. Effect of AMF inoculum availability (zero, medium, high) on the proportion of roots colonized by AMF in four milkweed species. Sample sizes range from 28-30 plants per plant species x AMF treatment. Bars display the mean ± 1SE.

Appendix B

Supplementary materials for Chapter III

Molecular analysis of AMF species in commercial inoculum

DNA was extracted from four separate samples of 250 mg of AMF inoculum using the PowerSoil DNA Isolation kit (MO BIO Laboratories, Inc.), following the instructions of the manufacturer. We used 5 µl of each DNA extract as the PCR template and AMF-specific primers designed to identify AMF to species level (Krüger et al. 2009). Specifically, we used the forward primer LROR and the reverse primer LSUmAr3 (Krüger et al. 2009). PCR was performed using 0.3125 U/µl ExTaq proofreading DNA polymerase (TaKara, Otsu, Japan) with 830 nM of each primer, 670 ng BSA/µl, 1.67x ExTaq buffer, 3.33 mM $MgCl_2$, 333 µM dNTPs in a 7.5 µl solution to which 5 µl of genomic DNA was added. Thermal cycling was done in an Eppendorf Mastercycler Pro S with the following conditions for PCR: 3 min initial denaturation at 94 ºC, then 35 cycles of 1 min denaturation at 94 ºC, 30s annealing at 56 ºC, and 1 min elongation at 72 ºC, and a 7 min final elongation. PCR products were cloned into a TOPO TA vector (Invitrogen) and transformed into Top 10 competent cells (Invitrogen). PCR was used to screen positive colonies using primers M13F and M13R provided by the TOPO TA cloning kit and following the manufacturer's instructions. PCR products from 11-14 clones were purified for each of the four inoculum samples (50 clones total) using ExoSAP (Promega) and sequenced using the M13F and M13R primers by the University of Michigan sequencing core using Sanger sequencing. The sequences were assembled and edited in Sequencher, and the identity of the clones determined using NCBI BLAST server. In doing so, the only AMF species identified was *Funneliformis mosseae*.

To confirm that *F. mosseae* was the only AMF species present in our inoculum, we also extracted DNA from approximately 70 mg flash-frozen plant roots of *A. curassavica*, *A. incarnata*, and *A. syriaca* inoculated with AMF using the DNeasy Plant Mini Kit (Qiagen) following the

instructions of the manufacturer. We used 5 µl of each DNA extract as the PCR template with the same primers (LROR, LSUmAr3) and PCR reaction mix and conditions as described previously. The PCR products were then purified with ExoSAP, and Sanger sequenced by the University of Michigan sequencing core. The sequences were assembled in Sequencher and used to query the NCBI BLAST server. We recovered only one clean sequence type from each plant sample, and that again blasted to *F. mosseae* for all plant samples, confirming that the AMF inoculum consisted predominantly of *F. mosseae*.

Effects of AMF-mediated plant traits on herbivore toxin sequestration and performance
Methods

To gain some insight into the phenotypic traits of plants through which AMF influenced herbivores, and largely to provide directions for future work, we assessed the effects of measured plant traits on herbivore performance and sequestration using multiple regression. Because herbivore and plant traits were measured from different groups of plants (see text for details), we calculated average values of plant and herbivore traits for each plant species x AMF treatment. This yielded 12 data points for herbivore performance measures (3 AMF treatments for each of 4 plant species) and 9 data points for herbivore sequestration measures (3 AMF treatments for each of 3 plant species; herbivores feeding on *A. incarnata* rarely sequestered cardenolides and were excluded from these analyses). We recognize that the 12 (or 9) data points are not independent, as we may expect AMF treatments or plant species to be more similar within than among treatment groups. Typically, such analyses would be conducted separately within each treatment group. However, with so few data points, we were unable to do this, and we were interested in assessing whether potential chemical mechanisms are general enough to operate across treatments. We therefore present the results of our analyses using only Information Theoretic (AICc) approaches and avoid presenting (spurious) *P*-values associated with our regressions.

Specifically, we used forward model selection based on AICc criteria to explore potential relationships among herbivore and plant traits (GLM SELECT, SAS 9.4). At each step in model selection, we verified that our plant traits (independent variables) were not correlated with one another. New variables were included in models only if they improved AICc scores by at least 2

(Burnham and Anderson 2002, Grossman et al. 2006). We ran models focusing only on those traits of plants and herbivores that were affected significantly by AMF (Tables 3.1 & 3.2).

Results

When we used AICc criteria to assess stepwise multiple regression models associating insect toxin sequestration and performance with those plants traits affected by AMF, our final models never included more than one significant predictor variable (Table B3.3). The concentration of cardenolides sequestered by aphids and caterpillars was positively associated with foliar cardenolide concentrations (Table B3.3, Figs. B3.2a,b). Similarly, the representation of lipophilic (non-polar) cardenolide compounds that caterpillars sequestered was positively associated with foliar cardenolide concentrations (Table B3.3, Fig. B3.2c). However, this pattern appears to be driven largely by differences among plant species in foliar cardenolide lipophilicity, not differences among AMF treatments. The average mass of individual aphids per plant was negatively associated with foliar cardenolide concentrations (Table B3.3, Fig. B3.2d), while the probability of caterpillar survival was positively associated with plant aboveground biomass (Table B3.3, Fig. B3.2e). Although aphid per capita growth rates (r) and the diversity of cardenolides sequestered by caterpillars were affected strongly by the availability AMF inoculum to their host plants (Table 3.2), they were not affected by any measured plant traits that were altered by AMF (Table B3.3).

Table B3.1. Number of aphid populations and individual caterpillars that sequestered cardenolides out of the total number of aphid populations and caterpillars per plant species x AMF treatment. Initial samples sizes were 15 aphid populations and 20 caterpillars (=replicate plants) per treatment combination. Only aphid populations that attained a mass of 1.0 mg or greater were included in the total, because we were not able to consistently detect cardenolides in smaller aphid populations, leading to the decreased total sample sizes. Some caterpillars escaped or died before the end of the experiment, leading to the decreased total sample sizes for caterpillars.

		AMF inoculum availability					
		Zero		Medium		High	
Herbivore	**Plant species**	Sequestered	Total	Sequestered	Total	Sequestered	Total
Aphid	*A. curassavica*	6	10	8	11	11	14
	A. incarnata	0	15	0	14	0	14
	A. latifolia	8	12	4	9	7	13
	A. syriaca	6	10	3	11	6	14
Caterpillar	*A. curassavica*	17	17	17	17	16	16
	A. incarnata	1	16	1	16	0	18
	A. latifolia	12	12	15	15	8	8
	A. syriaca	14	14	9	9	15	15

Table B3.2. Initial and final samples sizes per plant species x AMF treatment. Final sample sizes were smaller than initial because several caterpillars died or escaped before the end of the experiment and several samples were lost during processing and chemical analyses. In addition, not all aphid populations sequestered cardenolides (Table B3.1), further reducing the sample size for measures of aphid sequestration of cardenolides.

	Sample size per treatment	
	Initial	**Final range**
Plant traits		
Proportion AMF colonization	10	9-10
Foliar cardenolide concentration	20	18-20
Foliar cardenolide diversity	20	18-20
Foliar cardenolide polarity	20	18-20
Leaf toughness (SLM)	20	17-20
Latex exudation	20	18-20
Aboveground biomass	20	17-20
Foliar P concentration	10	9-10
Foliar C concentration	10	9-10
Foliar N concentration	10	9-10
Herbivore sequestration		
Aphid cardenolide concentration	15	3-11 (median=6)
Aphid cardenolide diversity	15	3-11 (median=6)
Aphid cardenolide polarity	15	3-11 (median=6)
Caterpillar cardenolide concentration	20	8-17
Caterpillar cardenolide diversity	20	8-17
Caterpillar cardenolide polarity	20	8-17
Performance		
Aphid r	15	15
Aphid individual mass	15	13-15
Caterpillar growth rate	20	8-18
Caterpillar ECI	20	8-18
Caterpillar CLA	20	8-18

Table B3.3. Partial unstandardized regression coefficients for variables retained in stepwise regression based on AICc criteria relating herbivore performance and sequestration measures that were affected by AMF to milkweed phenotypic traits. Columns contain coefficient values of predictors and model fit information for each herbivore sequestration or performance measure reported from the stepwise best-fit model. Herbivore performance and sequestration measures affected by AMF include natural log-transformed concentration of cardenolides sequestered by aphids (mg/g), natural log-transformed concentration of cardenolides sequestered by caterpillars (mg/g), diversity of cardenolides sequestered by caterpillars, natural log-transformed polarity of cardenolides sequestered by caterpillars, aphid per capita growth rate (r), individual dry mass per aphid (µg), and the probability of caterpillar survival. Milkweed traits affected by AMF include natural log-transformed foliar cardenolide concentration (mg/g), aboveground biomass (g), and foliar P concentration (%). Regressions were conducted on the means for each AMF x plant species combination (n=12 for herbivore performance measures and n=9 for sequestration measures; herbivores that fed on *A. incarnata* were excluded from analyses of sequestration). All regression analyses were performed with GLM SELECT in SAS 9.4.

	Predictor			**Model Fit**	
	Foliar cardenolide concentration	Aboveground biomass	Foliar P concentration	r^2	AICc
Aphid cardenolide concentration	0.20	-	-	0.7162	-32.2101
Caterpillar cardenolide concentration	0.92	-	-	0.8215	-9.7865
Caterpillar cardenolide diversity	-	-	-	0	-6.9799
Caterpillar cardenolide polarity	0.93	-	-	0.8899	-14.6343
Aphid r	-	-	-	0	-17.2299
Aphid individual mass	-18.35	-	-	0.5769	71.1207
Caterpillar survival	-	0.32	-	0.3299	-31.2657

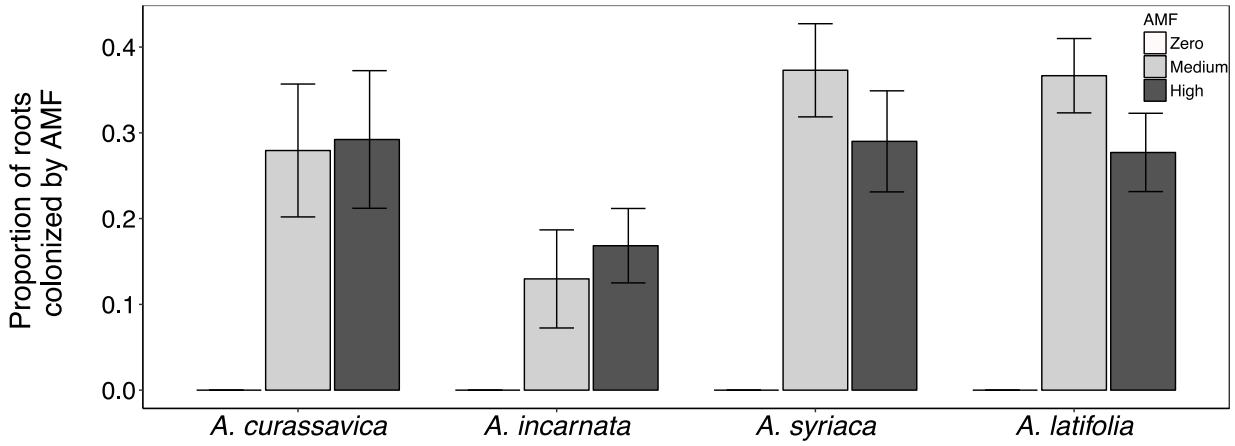

Figure B3.1. Effect of AMF inoculum availability on the proportion of roots colonized by AMF of four milkweed species. Sample sizes range from 9-10 plants per plant species x AMF treatment. Bars display the mean ± 1SE.

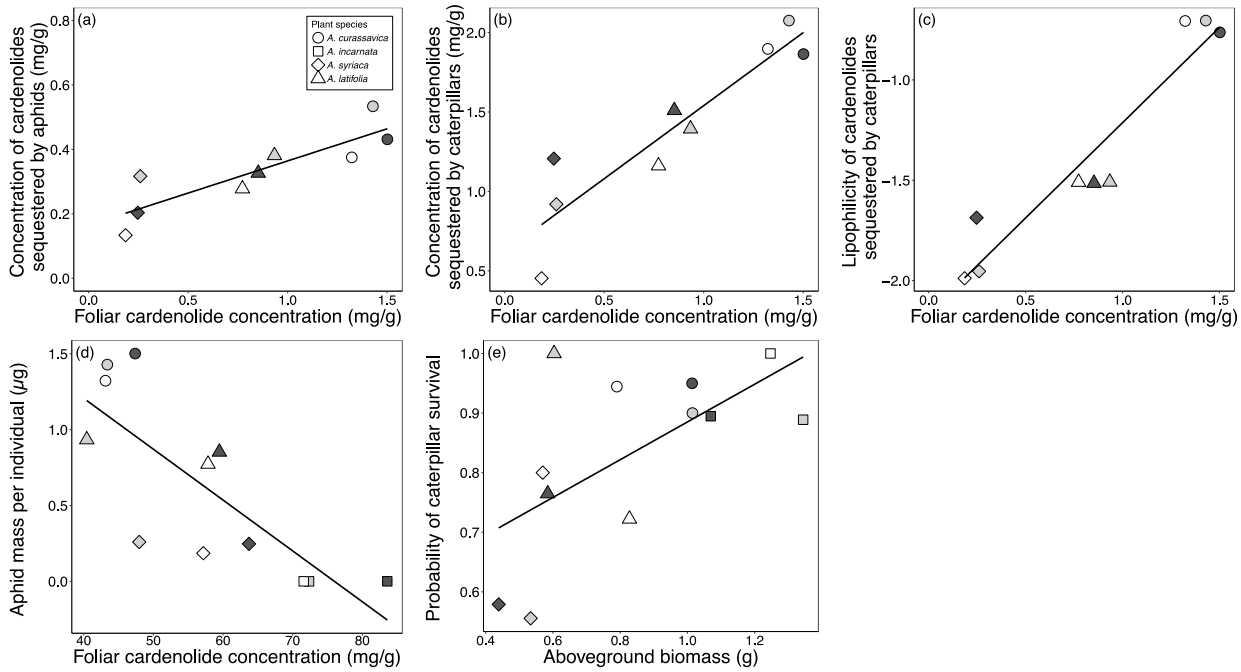

Figure B3.2. Relationships between measures of herbivore sequestration and performance and plant traits that were affected by AMF inoculum availability. Effects of natural log-transformed foliar cardenolide concentration on the natural log-transformed concentration of cardenolides sequestered by a) aphids and b) caterpillars, and on the c) natural log-transformed lipophilicity (non-polarity) of cardenolides sequestered by caterpillars. Effect of d) natural log-transformed foliar cardenolide concentration on the average mass of individual aphids per plant. e) Effect of plant aboveground biomass on the probability of caterpillar survival. Each point represents the mean herbivore trait regressed against the mean plant trait per plant species x AMF treatment combination, where black points represent high AMF plants, gray points represent medium AMF plants, and white points represent plants without AMF.

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Appendix C

Supplementary materials for Chapter IV

Table C4.1 Mean number of aphids ± standard error present during volatile collections on plants of each milkweed species under zero, medium, or high levels of AMF inoculum availability.

Plant species	**AMF**	**Aphid density**
A. curassavica	Zero	323 ± 29
A. curassavica	Medium	349 ± 41
A. curassavica	High	369 ± 35
A. incarnata	Zero	452 ± 27
A. incarnata	Medium	504 ± 50
A. incarnata	High	413 ± 42

Table C4.2. Effects of aphid abundance, plant species, AMF availability, plant species x AMF interaction, and PCA axes of environmental variables on the emission of volatile compounds that were affected significantly (P<0.05) by aphid feeding (see Table 4.2). Copaene, beta-cubebene, sesquiterpene 2, delta-cadinene, and unknown compound 10, were produced only by *A. incarnata*, so we did not assess differences in emissions of these compounds between milkweed species. **P < 0.001, *P < 0.05, †P < 0.1

	Aphid abundance	**Plant species**	**AMF**	**Plant species x AMF**	**PCA1**	**PCA2**
6-methyl-5-hepten-2-one	$F_{1,59.5}$=0.65 P=0.4235	$F_{1,22.1}$=0.18 P=0.6718	$F_{2,43.8}$=0.63 P=0.5367	$F_{2,44.1}$=0.08 P=0.9194	$F_{1,20}$=2.5 P=0.1292	$F_{1,21.4}$=4.08 P=0.0561†
cis-Ocimene	$F_{1,60.5}$=0.63 P=0.4317	$F_{1,22.2}$=8.45 P=0.0081**	$F_{2,43.8}$=0.79 P=0.4624	$F_{2,44.1}$=0.55 P=0.5782	$F_{1,20}$=0 P=0.9519	$F_{1,21.4}$=2.03 P=0.1683
Benzeneacetaldehyde	$F_{1,61.3}$=4.21 P=0.0446*	$F_{1,21.7}$=0.01 P=0.9239	$F_{2,43.2}$=0.07 P=0.9351	$F_{2,43.3}$=2.43 P=0.1003	$F_{1,19.3}$=0.58 P=0.4568	$F_{1,20.9}$=4.47 P=0.0466*
Copaene	$F_{1,22.1}$=0.05 P=0.8192		$F_{2,15}$=2.58 P=0.1091		$F_{1,6.3}$=0 P=0.9941	$F_{1,6.8}$=2.59 P=0.1529
beta-Cubebene	$F_{1,29.1}$=0.05 P=0.819		$F_{2,17.9}$=2.31 P=0.1278		$F_{1,8.2}$=0.01 P=0.9351	$F_{1,8.7}$=4.25 P=0.0705†
Sesquiterpene 2	$F_{1,23.5}$=0.02 P=0.8935		$F_{2,12}$=3.26 P=0.0738†		$F_{1,6.5}$=0.02 P=0.8854	$F_{1,7.1}$=4.48 P=0.0716†
delta-Cadinene	$F_{1,21.8}$=0.01 P=0.8935		$F_{2,22.2}$=1.88 P=0.1767		$F_{1,7.9}$=0.26 P=0.6255	$F_{1,8.5}$=7 P=0.0279*
Unknown compound 10	$F_{1,28.6}$=1.52 P=0.2278		$F_{2,19}$=3.67 P=0.045*		$F_{1,7}$=1.16 P=0.3169	$F_{1,7.5}$=0.04 P=0.8394